Marcos Leite Santoro

BUSCA POR POTENCIAIS MARCADORES GENÉTICOS NAS FASES INICIAIS DA ESQUIZOFRENIA

Tese apresentada à Universidade Federal de São Paulo – Escola Paulista de Medicina, para obtenção do Título de Doutor em Ciências.

São Paulo
2016

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por sempre me apoiarem em minha profissão.

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"...it ain't about how hard you hit,
it's about how hard you can get hit and keep moving forward,
how much you can take and keep moving forward.
That's how winning is done"
Rocky Balboa

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LISTA DE ABREVIATURAS e SÍMBOLOS

Lista de abreviaturas

Abreviaturas	**Significado**
PEP (ou FEP)	Primeiro episódio psicótico
GWAS	*Genome-wide association study*
EMR (ou UHR)	Estado Mental de risco para psicose
PRS	Escore poligênico de risco
SCZ	Esquizofrenia
DNA	Ácido desorribonucleico
RNA	Ácido ribonucléico
mRNA	RNA mensageiro
OR	*Odds Ratio*
LD	Desequilíbrio de ligação
N	Tamanho amostral (números inteiros)
pt	Limiar do valor de p (*p value threshold*)
5HT2a	Serotonina
GABA	Ácido gama-aminobutírico
RNAseq	sequenciamento de RNA
DNMT	DNA metiltransferase
CpG	Citosina, fosfato, guanina
5mC	5’-metilcitosina
MeCPs	proteínas ligantes de metil
SAM	S-adenosil-metionina
SNP	Polimorfismo de nucleotídeo único (*Single nucleotide polymorphism*)
TLDA	(*Taqman Low Density Array*)
DSM	Manual Diagnóstico e Estatístico de Transtornos Mentais (*Diagnostic and St* *Disorders*)
CAARMS	(*The Comprehensive Assessment of At-Risk Mental States*)
PGC	Consórcio internacional de genética psiquiátrica (*Psychiatric Genomics Consortiu*
PANSS	Escala de sintomatomas negativos e positivos (*Positive and Negative Syndrome*
BLIPS	(*Brief Limited Intermittent Psychotic Symptoms)*
APS	(*Attenuated Psychotic Symptoms)*
SCID	Entrevista Clínica Estruturada para o DSM (*Structured Clinical Interview for DSM*

CDSS	(*Calgary Depression Scale for Schizophrenia)*
GAF	(*Global Assessment of Functioning)*
CGI	(*Clinical Global Impression)*
PCR	Reação em cadeia da polimerase (*Polymerase Chain Reaction*)
ER	Retículo endoplasmático
ERAD	(*Endoplasmic-reticulum-associated protein degradation)*
PDs	Transtornos psiquiátricos (Psychiatric disorders)
CVCD	(*common disease-common variant)*
RVCD	(*common disease-rare variant)*
CNV	Variação no número de cópias (*copy number variation)*
AD	Doença de Alzheimer (Alzheimer disease)
BD	Transtorno Bipolar (Bipolar disorder)
ADHD	Transtorno de Défice de Atenção e Hiperatividade (*Attention deficit hyperactivity*
OCD	Transtorno obsessivo compulsivo (*Obsessive compulsive disorder*)
MDD	Transtorno depressivo maior (*Major depressive disorder)*
ASD	Autismo (*Autism spectrum disorder)*
AN	Anorexia Nervosa
TS	síndrome de Tourette (*Tourette syndrome)*
PTSD	Transtorno de estresse pós-traumático (*Posttraumatic stress disorder)*
mGlur	Receptor de glutamato metabotrópico (*metabotropic glutamate receptors)*
MHC	Complexo principal de histocompatibilidade (*major histocompatibility complex*)
df	Grau de Liberdade
QC	Controle de qualidae
SD	Desvio padrão

Genes

ACTB	actin, beta
AKT1	v-akt murine thymoma viral oncogene homolog 1
APOE	apolipoprotein E
BDNF	brain-derived neurotrophic factor
CACNA1C	calcium channel, voltage-dependent, L type, alpha 1C subunit
CACNB2	calcium channel, voltage-dependent, beta 2 subunit
CHRNA7	cholinergic receptor, nicotinic, alpha 7 (neuronal)
COMT	catechol-O-methyltransferase

DRD2	dopamine receptor D2
GABRR2	gamma-aminobutyric acid (GABA) A receptor, rho 2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCH1	GTP cyclohydrolase 1
GRIK3	glutamate receptor, ionotropic, kainate 3
NRG1	neuregulin 1
LPL	lipoprotein lipase
MAOA	monoamine oxidase A
MBP	myelin basic protein
PAFAH1B1	platelet-activating factor acetylhydrolase 1b, regulatory subunit 1 (45kDa)
RELN	reelin
RFX2	regulatory factor X, 2 (influences HLA class II expression)
S100B	S100 calcium binding protein B
SNCA	synuclein, alpha (non A4 component of amyloid precursor)
SOX10	SRY (sex determining region Y)-box 10
SYN2	synapsin II
TNF	tumor necrosis factor
TPH1	tryptophan hydroxylase 1
TUBB2A	tubulin, beta 2A class IIa
UFD1L	ubiquitin fusion degradation 1 like (yeast)
CUL3	cullin 3
SELENBP1	selenium binding protein 1
ADAMTS2	ADAM metallopeptidase with thrombospondin type 1 motif, 2
DGCR2	DiGeorge syndrome critical region gene 2
DICER1	dicer 1, ribonuclease type III
SIRT1	sirtuin 1
DAAM2	dishevelled associated activator of morphogenesis 2
EHF	ets homologous factor
DISC1	disrupted in schizophrenia 1
DROSHA	drosha, ribonuclease type III
DGCR8	DGCR8 microprocessor complex subunit
FAM63B	family with sequence similarity 63, member B
CNNM2	cyclin and CBS domain divalent metal cation transport mediator 2

LHPP	phospholysine phosphohistidine inorganic pyrophosphate phosphatase
CSMD1	CUB and Sushi multiple domains 1
CAMKMT	calmodulin-lysine N-methyltransferase
FAM124B	family with sequence similarity 124B
NDEL1	nudE neurodevelopment protein 1-like 1
CALHM1	calcium homeostasis modulator 1

RESUMO

A esquizofrenia é um transtorno psiquiátrico grave e incapacitante que acomete cerca de 1% da população. Ela é uma doença complexa caracterizada pela alteração em múltiplos genes de suscetibilidade, que atuam em conjunto com processos epigenéticos e ambientais. Uma vez diagnosticada a esquizofrenia, a maioria dos pacientes apresentam um prognóstico não favorável, sendo que poucos retornam às funções executivas normais após o primeiro episódio psicótico (PEP). Desta forma, o estudo de indivíduos em estados mentais de risco para psicose (EMR) é de grande importância para o entendimento da fisiopatologia da esquizofrenia, antes do estabelecimento da doença, bem como para encontrar marcadores relacionados à conversão para esquizofrenia. Igualmente importante, a investigação de indivíduos em seu PEP virgens de tratamento com antipsicóticos e antes da progressão da doença é extremamente útil para a compreensão da complexidade da esquizofrenia e seu tratamento. Considerando-se que a esquizofrenia é uma condição crônica, a progressão da doença e uso de medicação antipsicótica podem ser fatores confundidores na interpretação dos resultados de expressão gênica e de metilação do DNA. Nosso principal objetivo foi identificar em sangue periférico marcadores genéticos de risco, progressão e de resposta ao tratamento nas fases iniciais da esquizofrenia por diferentes enfoques (investigações genômicas, transcriptômicas e epigenômicas). Para tanto, investigamos 22 indivíduos em EMR, 66 pacientes em PEP em dois momentos: antes e após dois meses do início do tratamento com risperidona (FEP-2M), e ainda 67 controles saudáveis (sem histórico familiar ou pregresso de doenças psiquiátricas graves). Para melhor compreensão dos resultados, dividimos esta tese em dois estudos. No estudo 1 avaliamos a expressão de 12 genes candidatos pela técnica de TLDA (*Taqman Low Density Array*) em EMR, PEP antes do tratamento e controles. No estudo 2 utilizamos três medidas em larga escala: escore poligênico de risco para esquizofrenia (PRS – do inglês *Polygenic Risk Score*), transcriptoma e metiloma, todos por microarray em controles (N=60) e em pacientes PEP antes e após o tratamento (N=60). No estudo 1, nós encontramos dois genes diferencialmente expressos (*UFD1L* e *MBP*), sendo que o gene *UFD1L* apresentou uma expressão aumentada no grupo EMR em relação ao PEP e aos controles, sugerindo uma alteração específica dos indivíduos em EMR. No estudo 2, validamos o PRS e demonstramos que essa é uma medida que pode ser utilizada de forma eficaz na

população brasileira. Além disso, encontramos associações positivas no PEP entre o PRS e variáveis clínicas, de tal forma que quanto maior o PRS maior é o comprometimento no PEP. Porém, essas associações não são observadas após dois meses de tratamento com risperidona, reforçando a importância em se trabalhar com indivíduos em PEP virgens de tratamento. Por fim, identificamos alguns genes diferencialmente expressos e algumas regiões diferencialmente metiladas relacionadas com a doença (quando comparamos indivíduos em PEP e controles) e com a resposta ao tratamento com a risperidona (quando seguimos indivíduos em PEP antes e após o tratamento). Utilizando técnicas genômicas, transcriptômicas e epigenômicas fomos capazes de identificar em sangue periférico marcadores genéticos relacionados com a progressão e com as fases iniciais da esquizofrenia.

INTRODUÇÃO

1. INTRODUÇÃO

A presente tese foi derivada de quatro projetos de pesquisa (FAPESP 2010/08968-6; 2011/50740-5; 2014/50830-2; 2014/07280-1) que fazem parte da linha de pesquisa em Genética Psiquiátrica, a qual o aluno vem atuando desde 2006, junto ao grupo da Prof[a]. Dr[a]. Sintia Iole Belangero cujo principal objetivo é identificar potenciais marcadores genéticos de risco, progressão e doença. Além desses quatro auxílios, um quinto (Fapesp 2012/50316-1) foi bastante importante para a execução de um dos artigo de relacionados a essa tese (Santoro et al., 2016 - artigo de revisão). Para o nosso conhecimento, os estudos que compõem essa tese de doutorado foram os primeiros a I) verificar a expressão gênica em sangue de uma coorte de indivíduos em estado mental de risco para esquizofrenia (EMR), II) validar o escore poligênico de risco para esquizofrenia (PRS) em uma amostra brasileira, III) correlacionar o PRS com múltiplas variáveis clínicas em pacientes em primeiro episodio psicótico (PEP) virgens de tratamento com antipsicótico e IV) após o tratamento com Risperidona. Além disso, uma das metas do estudo foi avançar no conhecimento em bioinformática, aprendendo a utilizar as ferramentas para análise de dados em larga escala.

A seguir a tese apresentará as seguintes seções: “Revisão da literatura” que incluirá o embasamento necessário para o entendimento da importância desse estudo bem como uma revisão atualizada dos últimos achados relacionados com a tese; “Objetivos” no qual abordaremos o objetivo geral e os específicos; “Artigos científicos” (quatro artigos científicos derivados dessa tese, sendo dois publicados, um submetido e outro em preparo com o aluno como primeiro autor) em substituição aos itens ‘Casuística e Métodos’, ‘Resultados’ e ‘Discussão’ de uma tese tradicional, uma vez que cada um apresenta particularidades relacionadas a esses itens; “Limitações” do trabalho e “Conclusões gerais” do estudo.

Os dados gerados a partir dessa tese são originais e permitirão uma maior compreensão das bases genéticas da esquizofrenia, bem como a importância em se estudar as fases iniciais dessa doença.

OBJETIVOS

2 OBJETIVOS

2.1 Objetivo Geral

Identificar em sangue periférico potenciais marcadores de risco, de progressão e de resposta ao tratamento nas fases iniciais da esquizofrenia.

2.2 Objetivos do estudo 1

Encontrar genes relacionados ao estado mental de risco (EMR) e para a progressão da psicose, comparando a expressão de 12 genes candidatos em sangue periférico entre indivíduos em EMR (N=22) e controles (N=67) e entre indivíduos em EMR e pacientes em PEP (N=66).

2.3 Objetivos do estudo 2

Identificar em sangue periférico potenciais marcadores de risco, progressão e resposta à risperidona utilizando uma coorte longitudinal de pacientes em PEP virgens de tratamento com antipsicóticos (N=60) e um grupo controle (N=60), utilizando análises genéticas em larga escala (genômicas, transcriptômicas e epigenômicas).

2.3.1 Objetivos Específicos

- Validar o escore poligênico de risco para esquizofrenia (PRS) na população brasileira.

- Correlacionar o PRS com variáveis clínicas de gravidade, sintomatologia e resposta ao tratamento em pacientes em PEP antes e após o tratamento.

- Identificar genes e regiões diferencialmente expressas e metiladas nos estágios iniciais da esquizofrenia;

- Identificar genes e regiões diferencialmente expressas e metiladas relacionadas ao tratamento com risperidona.

REVISÃO DA LITERATURA

3 REVISÃO DA LITERATURA

3.1 **Enunciado do problema**

A esquizofrenia é um transtorno psiquiátrico grave que acomete cerca de 1% da população ao longo da vida (Kessler et al., 2005; Saha et al., 2005). Os indivíduos geralmente são acometidos no auge do seu potencial produtivo e, embora o curso da doença não seja uniformemente negativo como normalmente se pensa, mais de 50% dos indivíduos com esquizofrenia tem problemas psiquiátricos intermitentes e cerca de 20% têm sintomas crônicos e debilitantes (Barbato, 1998). O desemprego é incrivelmente alto nesse grupo chegando a 90% (Marwaha and Johnson, 2004; Kooyman et al., 2007), ao mesmo tempo, a expectativa de vida é reduzida com significativa morbidade (Goff et al., 2005) e mortalidade (Saha et al., 2007) associada à doença. Segundo dados da Organização Mundial da Saúde, a esquizofrenia é a 3ª principal causa de anos perdidos de vida ajustados por incapacidade (*Disability-Adjusted Life Year* – DALY) na população jovem de 10-24 anos (Gore et al., 2011). Dentre os transtornos mentais, ela representa um dos principais custos direto com tratamento, com cerca de 1,3 a 2,5% do total de custos em saúde em países ocidentais (Rossler et al., 2005).

A esquizofrenia é uma doença multifatorial, ou seja, diversos fatores ambientais e genéticos (poligênicos) contribuem para a doença. A sintomatologia da doença é igualmente complexa, composta por sintomas positivos (alucinações e delírios), sintomas negativos (embotamento afetivo, alogia e avolição) e sintomas cognitivos (déficit de atenção, de memória e de funções executivas). Na última década vimos avanços significativos na aplicação da genômica, epidemiologia e neurociência para pesquisa em esquizofrenia. Porém, dois dos maiores desafios ainda são compreender as causas e fisiopatologia da doença, bem como desenvolver novos tratamentos e terapias efetivos para controlar os sintomas ou até mesmo evitar o estabelecimento da doença.

3.2 **Tratamento da esquizofrenia**

A esquizofrenia é uma doença que requer manutenção do tratamento com antipsicóticos por um longo período na vida do paciente (Uchida et al., 2008). Em 1952, Delay e Deniker demonstraram que a clorpromazina era efetiva para o tratamento da

esquizofrenia (Rosenbloom, 2002). Atualmente os antipsicóticos são divididos em duas classes: típicos e atípicos. Os antipsicóticos típicos são basicamente antagonistas dos receptores D2/D3 de dopamina, já os antipsicóticos atípicos apresentam um perfil bioquímico mais complexo e possuem afinidade por receptores de diversos neurotransmissores como a serotonina, histamina, acetilcolina entre outros (Lopez-Munoz and Alamo, 2011).

Uma recente metanálise demonstrou que as duas classes de antipsicóticos são semelhantes em relação à eficácia no controle dos sintomas positivos da esquizofrenia, mas diferem em relação ao perfil de efeitos adversos desencadeados (sintomas extrapiramidais, discinesia tardia, ganho de peso) (Crossley et al., 2010). Os antipsicóticos atípicos apresentam menor chance de induzir esses sintomas (Kapur and Seeman, 2001) e ainda podem melhorar os sintomas negativos e cognitivos da esquizofrenia (Woodward et al., 2005).

Entre os antipsicóticos mais utilizados na clínica está a risperidona. Esta droga é considerada um antipsicótico atípico por possuir alta afinidade pelos receptores de 5HT2a (antagonismo), porém em altas doses pode atingir uma taxa de ocupação de D2 maior que 80% (semelhante aos antipsicóticos típicos) e apresentar sintomas extrapiramidais (Klemp et al., 2011; Uchida et al., 2011). Esta droga tem sido utilizada em baixas doses para pacientes em primeiro episódio psicótico (PEP) (2001) e em pacientes com depressão que apresentam alto risco de suicídio ou refratários ao tratamento (Reeves et al., 2008; Wright et al., 2013).

3.3 **Esquizofrenia como uma doença do neurodesenvolvimento**

Apesar do impacto da esquizofrenia para os pacientes, familiares e o sistema de saúde, além de ser alvo de estudos há mais de 100 anos, suas causas ainda não foram completamente esclarecidas. Algumas hipóteses foram levantadas sobre a etiologia da esquizofrenia, dentre elas a do neurodesenvolvimento. Nela é proposto que a doença é resultado de um processo de neurodesenvolvimento alterado que antecede o acometimento de sintomas clínicos e é causado por uma combinação de fatores genéticos e ambientais (Leroy *et al.*, 2001; Caspi *et al.*, 2005). Essas alterações podem predispor a ativação de circuitos neurais patológicos durante a adolescência ou início da vida adulta, levando à emergência de sintomas positivos, negativos ou ambos (Brown et al., 2004). A Figura 1 exemplifica as fases da esquizofrenia.

Atualmente, a esquizofrenia é vista como uma doença do neurodesenvolvimento, apresentando uma trajetória clínica e neurobiológica desde a sua primeira manifestação sintomática até as fases crônicas e refratárias da doença (Lewis and Levitt, 2002; Owen et al., 2011). Dessa forma, a perspectiva moderna da psiquiatria destaca a necessidade de desenvolver e implementar estratégias de prevenção para a doença.

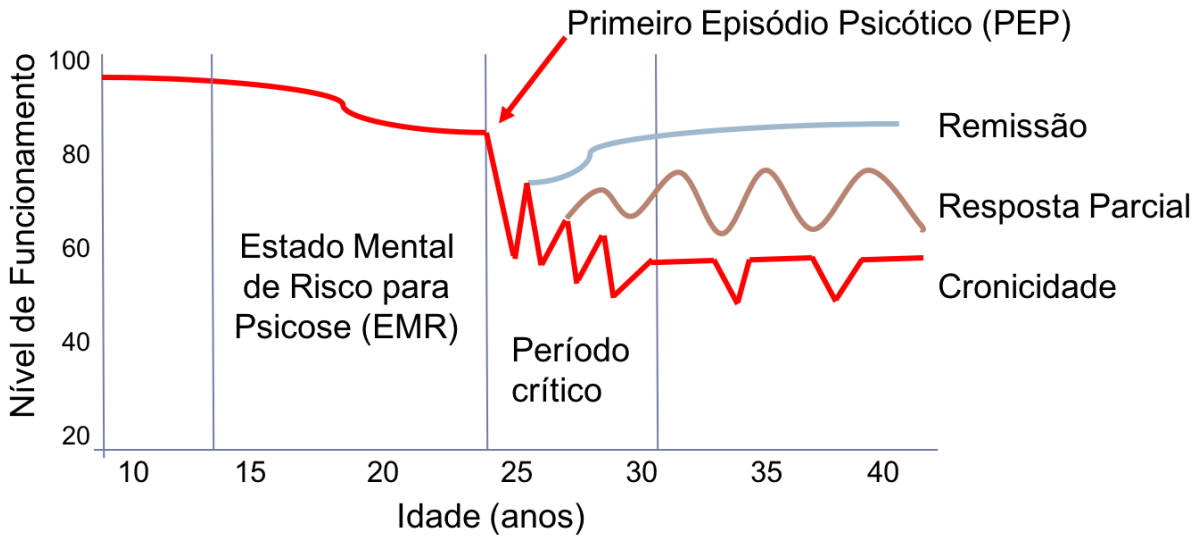

Figura 3. Gráfico representativo da evolução da esquizofrenia com relação ao nível de funcionamento.

3.3.1 Estado Mental de risco para psicose

As observações anteriores sugerem que estágios iniciais da doença são uma oportunidade para medidas preventivas e intervenções na doença. O início dos sintomas formais da esquizofrenia é geralmente precedido por uma 'fase prodrômica' que demonstra início iminente da doença. Os sintomas e comportamentos prodrômicos incluem: sintomas positivos atenuados (ilusões, ideias de referência e pensamento mágico); sintomas de humor (ansiedade e irritabilidade); sintomas cognitivos (distração e dificuldades de concentração); e isolamento social ou comportamentos obsessivos (McGlashan and Johannessen, 1996). O pródromo, no entanto, só pode ser definido de forma retroativa, ou seja, em pacientes que já possuem a esquizofrenia. Apesar dos avanços significativos na caracterização e operacionalização dos critérios para definir o pródromo em esquizofrenia, a identificação confiável de indivíduos em risco que realmente convertem para a doença ainda é um grande desafio. A sintomatologia desse grupo de risco se confunde com outros transtornos psiquiátricos e em sua

grande maioria os indivíduos em risco não convertem para a esquizofrenia (Lim et al., 2015) Fusar-Poli et al., 2012).

Em 1996, dois estudos desenvolveram critérios para identificação de indivíduos em Estado Mental de Risco para psicose (EMR). Estes critérios envolvem uma combinação de alterações cognitivas, comportamentais, afetivas ou psicóticas inespecíficas e em intensidade ou frequência insuficiente para ser considerado um transtorno mental, em adição a risco genético e declínio funcional (McGlashan and Johannessen, 1996; Yung and McGorry, 1996). Os critérios de conversão requerem tipicamente a ocorrência de pelo menos um dos sintomas psicóticos positivos durante várias vezes por mais de 1 semana (Yung et al., 2005). Contudo, evidências indicam que a maioria dos indivíduos identificados como EMR não irão desenvolver esquizofrenia (Simon et al., 2011). Uma metanálise mostrou que a conversão para a psicose varia de 18%, com 6 meses de acompanhamento, até 36% após 3 anos de seguimento (Fusar-Poli et al., 2012). Dessa forma, uma melhor precisão da predição ainda é necessária

A predição de desenvolvimento de doença em indivíduos em estágios prodrômicos é um campo incipiente na pesquisa em Psiquiatria. Alguns autores fizeram tentativas de melhorar o poder preditivo agregando medidas como uso de substâncias, distorções de pensamento, suspeição, hereditariedade e declínio no funcionamento. Nesse sentido, Cannon et al. (2008) reportaram um estudo de seguimento de dois anos e meio de 291 indivíduos em EMR e propuseram a combinação de três variáveis identificadas como preditoras de psicose como forma de aumentar o valor preditivo positivo quando comparada aos critérios de pródromos tomados isoladamente (68 a 80% *vs*. 35%, respectivamente) (Cannon et al., 2008). Apesar de esses dados serem promissores, a capacidade preditiva ainda é limitada, ficando evidente a necessidade de replicação desses resultados bem como a identificação de melhores preditores para que não haja intervenções desnecessárias em indivíduos jovens, evitando sintomas adversos e o estigma.

Até o momento, poucos estudos verificaram alterações genéticas em indivíduos em EMR. Um estudo multidisciplinar recente mostrou que dados de testes neurocognitivos e de neuroimagem foram bons preditores para diferenciar pacientes em primeiro episódio psicótico (PEP) e indivíduos em EMR, por outro lado, polimorfismos genéticos não foram capazes de predizer os grupos (Pettersson-Yeo et al., 2013). Contudo, levando em consideração que o EMR é um “estado” e que esses

indivíduos podem ou não converter para esquizofrenia, seria interessante verificar as alterações ao nível transcriptômico desses indivíduos e não alterações genômicas estáticas.

3.3.2 Primeiro Episódio Psicótico

O desenvolvimento de sintomas psicóticos francos marca o início formal do PEP da esquizofrenia, embora isso seja diagnosticado até que o paciente procure ou seja trazido para a atenção médica.

Os primeiros 2-5 anos pós-diagnóstico de PEP podem ser considerados como um período crítico que influencia o curso da doença a longo prazo. Durante esse período as deteriorações sintomática e psicossocial progridem rapidamente (Birchwood et al., 1998). As fases precoces da doença, incluindo o tempo de psicose não tratada, estão associadas à perda de contato com a família e amigos (Jeppesen et al., 2008), tentativas de suicídio (Nordentoft *et al.*, 2004; Large *et al.*, 2009; Nordentoft *et al.*, 2011), comorbidade com uso de substâncias e criminalidade (Nielssen et al., 2012; Stevens et al., 2012).

Apesar de alguns indivíduos recuperarem sintomaticamente após o PEP, a maioria deles continua a ter um ou mais episódios subsequentes sob a forma de recaídas psicóticas (Lieberman, 1993). Este processo de recaídas leva muitos pacientes a um curso crônico da doença, adquirindo perturbações e déficits persistentes. A possibilidade de alterar a evolução da doença, levou ao desenvolvimento e estabelecimento de intervenções precoces especializadas para o paciente em PEP, que se intensificaram internacionalmente nas últimas duas décadas (Nordentoft et al., 2014).

Para a esquizofrenia, existe uma grande heterogeneidade de resposta ao tratamento, com uma menor proporção (20-50%) de pacientes que remitem ou que tem uma melhora significativa, comparado com a maioria que apresenta múltiplos episódios e um declínio acentuado (Bleuler, 1978; Ciompi, 1980; Huber *et al.*, 1980; Shepherd *et al.*, 1989; Harding *et al.*, 1992; van Os *et al.*, 1996). A maior parte dos estudos em esquizofrenia incluem pacientes em diferentes estágios da doença, com uma representação excessiva de pacientes crônicos e refratários ao tratamento (Shepherd *et al.*, 1989; Keshavan and Schooler, 1992; Lieberman *et al.*, 1996; Birchwood *et al.*, 1998; Riecher-Rossler and Rossler, 1998) e uma restrição de pacientes que

melhoraram ou remitiram, mas que receberam um diagnóstico inicial de esquizofrenia (Davidson and McGlashan, 1997). Assim, a heterogeneidade da amostra com predominância de pacientes em estágio mais avançados da doença direciona as pesquisas para pacientes com uma pior evolução da esquizofrenia, atribuindo as características e cursos dos pacientes de pior prognóstico para toda a população com a doença.

Tanto a identificação quanto o manejo precoce das doenças mentais são mencionados como uma área de prioridade no plano de ação da saúde mental de 2013 a 2020 (World Health Organization, 2013; Nordentoft *et al.*, 2014). Dessa forma, a investigação dos pacientes no início da esquizofrenia, e particularmente durante o EMR e o PEP podem auxiliar no entendimento da doença e de sua progressão, além de ser mais generalizável do que os estudos em pacientes crônicos.

3.4 **Bases genéticas da esquizofrenia**

A pesquisa em neurociência tem mostrado que os transtornos psiquiátricos mais graves são de base do desenvolvimento neurológico resultante de uma interação específica de fase dinâmica de fatores biológicos e ambientais que levam à interrupção na montagem normal do cérebro (Stachowiak et al., 2013). Como dito anteriormente, a esquizofrenia apresenta uma etiologia multifatorial, em que fatores de risco genéticos e ambientais interagem para o desenvolvimento da doença. Como prova disso, a história familiar e a exposição a fatores ambientais como complicações obstétricas, infecções maternas e uso de maconha têm demonstrado influenciar o risco para o desenvolvimento de esquizofrenia (McDonald and Murray, 2000; Lewis and Levitt, 2002). Estudos de trios demonstraram que parentes de primeiro grau de pacientes com esquizofrenia têm um risco de 10% em apresentar a doença (dez vezes maior do que o risco na população geral de 1%), já para irmãos gêmeos monozigóticos a concordância da doença é de 40-50%. Com base nestes estudos, estimou-se a herdabilidade da esquizofrenia pode chegar a 80% (Berrettini, 2000; McGuffin *et al.*, 2003; Sullivan *et al.*, 2003; Lichtenstein *et al.*, 2009).

3.5 **Estudos de associação genética em larga escala (GWAS) em esquizofrenia**

Basicamente, um estudo de associação genética em larga escala (GWAS, do inglês *Genome-wide Association Study*) é uma análise de um conjunto de variantes

genéticas (milhares ou mesmo milhões de variantes) em diferentes grupos de indivíduos para verificar se alguma destas variantes está associada a determinado fenótipo. Em sua grande maioria, essas variantes genéticas são polimorfismos de nucleotídeo único (SNPs), os quais, por definição, são substituições de apenas um nucleotídeo e que possuem uma frequência na população para o alelo raro de pelo menos 1%.

O avanço dos GWASs tem sido bem-sucedido em descobrir novos genes e vias para diversas doenças. Como exemplo, estudos encontraram alvos para hipertensão e diabetes, abrindo oportunidade para novos tratamentos e diagnósticos precoces (Manolio, 2010). Por outro lado, tornou-se evidente que nenhuma variante sozinha é capaz de explicar o risco para a maioria dos transtornos psiquiátricos, mas sim centenas de variantes conferem pequenos efeitos para o risco em desenvolver estas doenças (Glessner et al., 2010a; Klein et al., 2010; McClellan and King, 2010).

Ao longo dos últimos anos, dezenas de GWAS encontraram centenas de variantes associadas com a esquizofrenia (Chen et al., 2015). O último GWAS realizado pelo PGC sedimentou o conceito de doença poligênica para a esquizofrenia. Com uma amostra de 36.989 pacientes e 113,075 controles saudáveis, foram identificadas 83 novas regiões independentemente associadas com a doença. Entre os resultados desse estudo, os autores ainda estimaram que uma nova variante comum deve ser identificada para cada 250 casos e 250 controles adicionados para o tamanho da amostra.

Além identificar regiões genômicas associadas com a etiologia da doença, o GWAS pode ser aplicado para o reconhecimento de endofenótipos. No caso da esquizofrenia, esse tipo de estudo tem sido aplicado para identificar variantes associadas com a resposta aos antipsicóticos ou mesmo relacionadas com efeitos adversos (Alkelai et al., 2009; Lavedan et al., 2009; Volpi et al., 2009).

3.5.1 Escore poligênico de risco para esquizofrenia

Em 2009, Purcell e colaboradores descreveram a possibilidade em se utilizar uma pontuação poligênica a partir de qualquer GWAS. Este estudo, entre outros resultados, demonstrou um compartilhamento genético entre a esquizofrenia e o transtorno bipolar. Curiosamente, eles notaram que esta partilha foi elevada, não só para as variantes estatisticamente significantes ($p<5x10^{-8}$), mas também para as variantes com índice de significância maiores (até $p=0.5$). Levando em conta a

premissa de que essas doenças psiquiátricas são poligênicas e que essas variantes apresentam tamanho de efeito muito baixo, eles argumentaram que mesmo essas variantes com maior índice de significância deveriam atuar no risco da doença (Purcell et al., 2009; Ruderfer et al., 2014; Franke et al., 2016).

Com o aumento exponencial do tamanho da amostra dos consórcios internacionais, esta ferramenta tornou-se ainda mais útil e tem se consolidado nos últimos dois anos no campo da genética psiquiátrica. Desta forma, com os resultados de um GWAS como uma base (*summary statistics*) é possível calcular em uma outra amostra independente um escore de risco de doença para cada indivíduo. O escore poligênico de risco (PRS, do inglês *polygenic risk score*) usa um valor de p (não necessariamente o limiar conservador do GWAS de p $<5x10^{-8}$) como um limiar para selecionar quais variantes da amostra base serão utilizadas. Em seguida, a partir do tamanho do efeito (ou *odds ratio*) da variante no GWAS base, cada indivíduo recebe uma pontuação de acordo com o número de alelos de risco que ele possui. Isso é feito para todas as variantes selecionadas, de tal forma que ao fim da soma cada indivíduo terá um escore representando o risco para determinado fenótipo (Purcell et al., 2009; Dudbridge, 2013). A Figura 2 exemplifica essa metodologia.

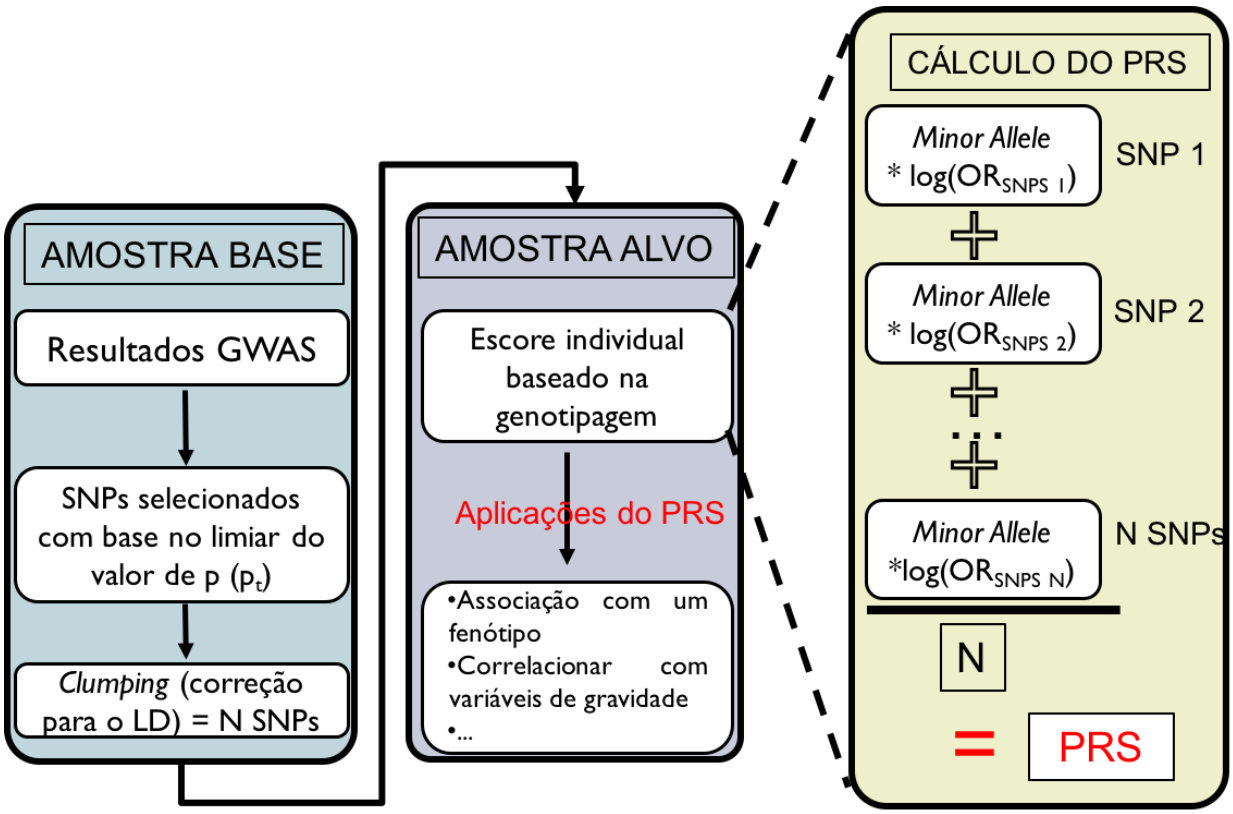

Figura 4. Cálculo do PRS. Passos e amostras necessárias para o cálculo do PRS. Adaptado de Purcell et al., 2009.

A grande vantagem do PRS, se estimado de um GWAS com alto poder e representativo da população, é que não é mais preciso grandes tamanhos amostrais para se extrair uma informação confiável de uma genotipagem em larga escala, já que esse escore é individual (Dudbridge, 2013). A aplicabilidade do PRS é ampla, com esta pontuação podemos desde verificar a consistência de fenótipo-genótipo entre diferentes populações, até utilizar este escore como uma variável e correlacionar com outras características clínicas. Com esse potencial, nos próximos anos o PRS pode tornar-se um passo fundamental para qualquer estudo que envolva risco genético para doenças poligênicas.

O PRS tem apresentado resultados interessantes, como a associação entre os cinco principais transtornos psiquiátricos (Demirkan et al., 2011; Cross-Disorder Group of the Psychiatric Genomics, 2013; Cross-Disorder Group of the Psychiatric Genomics et al., 2013). Para esquizofrenia, o PRS foi correlacionado com variáveis quantitativas, como a gravidade de sintomas (Derks et al., 2012) e da atividade pré-frontal (Walton et al., 2013).

3.6 **Expressão gênica em sangue periférico**

Uma das abordagens para verificar a estabilidade de vias de neurotransmissão e o funcionamento dos genes relacionados a neuroplasticidade e neurodesenvolvimento é o estudo de expressão gênica. Considerando que o DNA é transcrito para RNA, que por sua vez pode ou não ser traduzido em proteína, podemos considerar que a expressão gênica é um fenótipo intermediário entre a sequência gênica e a doença. Assim, estudos que avaliam a quantidade de RNA e/ou proteínas são essenciais para o entendimento do papel de variantes genéticas na suscetibilidade à esquizofrenia (de Jong et al., 2012), além de refletirem as múltiplas interações genéticas e ambientais. Adicionalmente, a maior parte das variantes genéticas que conferem risco à doença não ocorrem em regiões codificantes, não alterando a estrutura proteica, mas podendo influenciar na sua expressão.

Alguns estudos utilizando *microarrays* em tecidos cerebrais *post mortem* de pacientes com esquizofrenia já relataram diferenças de expressão em genes pertencentes às vias de mielinização, GABAérgicas, glutamatérgicas, de sinapse, de mitocôndrias, de resposta imune e de estresse (Sequeira et al., 2012). Um estudo recente de sequenciamento de RNA em larga escala (RNAseq) em amostras *post-mortem* de hipocampo, identificou 144 genes diferencialmente expressos entre

pacientes com esquizofrenia e controles, muitos deles relacionados com as vias de inflamação e resposta imune (Hwang et al., 2013).

Entretanto, diversos fatores confundidores podem ter um efeito significativo na expressão gênica em tecido cerebral *postmortem*, mascarando e enviesando esses estudos, tais como a idade, pH, gênero, qualidade do mRNA (Sequeira et al., 2012) e tempo de tratamento com antipsicóticos (Molteni et al., 2009). Devido a essas limitações, tem sido proposto que a análise de expressão gênica em sangue periférico pode ser um instrumento útil para detectar a presença de marcadores de progressão em vários distúrbios neuropsiquiátricos (Middleton *et al.*, 2005; Kumarasinghe *et al.*, 2013; Wankerl *et al.*, 2014).

Embora a expressão gênica em sangue seja moderadamente correlacionada com a expressão gênica em tecido cerebral (Sullivan *et al.*, 2006; Jasinska *et al.*, 2009; Cai *et al.*, 2010; Rollins *et al.*, 2010), diversos estudos têm sugerido que a primeira pode ser útil para auxiliar no diagnóstico de doenças mentais (Middleton *et al.*, 2005; Tsuang *et al.*, 2005; Takahashi *et al.*, 2010; de Jong *et al.*, 2012; Frau *et al.*, 2012; Maschietto *et al.*, 2012) ou na classificação de subtipos (Bowden et al., 2006) ou na sintomatologia, como alucinações e delírios (Bousman et al., 2010; Kurian et al., 2011).

Assim, alguns estudos têm observado alterações de expressão em tecidos periféricos de genes relacionados com a esquizofrenia (Glatt et al., 2005; Zhang et al., 2008)(Ota et al., 2014). Cinco estudos que avaliaram o perfil de expressão gênica em sangue periférico de paciente com esquizofrenia por meio de *microarrays* (em larga escala), identificaram alterações na expressão de genes que estão envolvidos em função imune, inflamação (de Jong et al., 2012; Gardiner et al., 2013; Kumarasinghe et al., 2013), doenças infecciosas (de Jong et al., 2012; Kumarasinghe et al., 2013), função hematológica, doenças neurológicas (de Jong et al., 2012), transcrição / processamento de RNA, ubiquitina, metabolismo de lipídio / glicose / proteína, transdução de sinal, citoesqueleto (Kuzman et al., 2009), adesão celular, desenvolvimento do sistema nervoso, transporte iônico (Takahashi et al., 2010), entre outras funções. Um estudo utilizando RNAseq confirmou alterações em genes relacionados ao sistema imune (Xu et al., 2012).

Contudo, o tratamento com antipsicóticos é um fator confundidor que deve ser considerado, uma vez que o uso de medicação por longos períodos pode influenciar nos resultados. Utilizando a técnica de *microarray,* Mamdani et al. (2013) observaram que 210 transcritos apresentaram diferença de expressão entre pacientes que

receberam risperidona ou olanzapina (Mamdani et al., 2013). Além do tratamento, a própria cronicidade e evolução da doença podem interferir nos resultados, uma vez que a expressão de alguns genes pode diferir entre os estágios iniciais e tardios da doença.

Até o momento poucos estudos verificaram a expressão gênica no sangue de pacientes no PEP. Dois trabalhos do nosso grupo (Ota et al., 2014a; Ota et al., 2014b) compararam a expressão de 12 genes envolvidos na neurotransmissão ou neurodesenvolvimento entre 51 pacientes em PEP virgens de tratamento e 51 controles (Ota et al., 2014b) e entre 44 pacientes em PEP antes e após 2 meses de tratamento com o antipsicótico atípico risperidona (Ota et al., 2014a). No primeiro trabalho, encontramos uma redução da expressão do gene *GCH1* nos pacientes em comparação aos controles, a qual foi mantida após o tratamento. Já no segundo, identificamos por meio de uma análise longitudinal uma redução da expressão do gene *GABRR2* após o tratamento com o antipsicótico, indicando que risperidona também pode interferir na expressão gênica.

Até o momento, alguns estudos investigaram expressão gênica em sangue total de pacientes virgens de tratamento e em PEP, relatando diferenças em genes envolvidos na transcrição e processamento de RNA, ubiquitina, metabolismo de lipídio / glicose / proteína, transdução de sinal, citoesqueleto e fatores de crescimento (Zhang et al., 2008; Kuzman et al., 2009; Sainz et al., 2013; Crespo-Facorro et al., 2014). Alguns trabalhos mostraram que os genes *NRG1* e *DAAM2* poderiam ser potenciais marcadores de progressão e/ou resposta ao tratamento, uma vez que os níveis de expressão retornaram ao normal após a remissão (Zhang *et al.*, 2008; Kuzman *et al.*, 2009).

A comparação da expressão gênica entre indivíduos em EMR e em PEP tem o potencial de fornecer novos *insights* sobre a neurobiologia da esquizofrenia, desvendando o mecanismo de conversão de um estado de risco para uma doença potencialmente grave. Além disso, a investigação de potenciais marcadores associados ao desenvolvimento de psicose pode aumentar a precisão do reconhecimento daqueles indivíduos que realmente estão em EMR.

O estudo de expressão gênica em tecido periférico de pacientes em EMR e PEP também permite identificar marcadores de traço e de estado. Os marcadores de traço referem-se aos processos biológicos ou comportamentais que exercem um papel, possivelmente causal, na predisposição de uma doença psiquiátrica, enquanto que os marcadores de estado se referem mais às manifestações clínicas dos pacientes.

Tipicamente, embora não necessariamente, um marcador de traço é duradouro, enquanto que o de estado é transiente (Chen et al., 2006).

3.7 **Epigenética na esquizofrenia**

O termo *epigenética* refere-se a modificações químicas e físicas do DNA e da cromatina que regulam a atividade funcional do genoma, como a expressão gênica, sem mudar a sequência de nucleotídeos (Turecki et al., 2014). Essas alterações são responsáveis pelo controle transcricional, por meio de repressão de genes desnecessários em algumas células. Diversas evidências demonstraram que eventos epigenéticos estão envolvidos no desenvolvimento embrionário, no fenômeno de compensação de dose, na regulação da expressão gênica e na estabilidade genômica (Park and Kuroda, 2001; Li, 2002; Margueron and Reinberg, 2010).

Dentre as modificações epigenéticas, a mais estudada é a metilação do DNA, que foi muito investigada em situações de resposta ao estresse. Liu et al. (1997) observaram uma redução de expressão do gene receptor de glicocorticóide (*Nr3c1*) em hipocampo de ratos criados por mães menos cuidadosas (que lambem menos a sua cria, modelo *licking / grooming*-LG) e também na prole destas mães, que apresentaram maior metilação do gene *Nr3c1 e* resposta ao estresse (Liu et al., 1997). Em seguida, um estudo do mesmo grupo identificou um aumento da metilação na região promotora desse gene, indicando uma relação causal entre o estado epigenômico, os níveis de expressão gênica e o efeito materno na resposta ao estresse desses filhotes (Weaver et al., 2004).

Genótipos individuais em *loci* específicos podem resultar em diferentes padrões de metilação do DNA. Estes loci controladores de traços quantitativos da metilação (meQTLs) influenciam na metilação de amplas regiões genômicas e podem ser a base das associações entre SNPs ou das interações gene-ambiente (Smith et al., 2014) e foram encontradas em todo o genoma de diferentes tecidos (Kerkel et al., 2008; Schalkwyk et al., 2010). Desta forma, efeitos genéticos sobre a metilação podem atuar como um mecanismo pelo qual os padrões de metilação podem ser transmitidas através das gerações (Drong et al., 2013). Esse mecanismo é de grande interesse para a compreensão das doenças complexas, onde fatores genéticos e epigenéticos parecem convergir para o desencadeamento dos sintomas.

No caso da metilação do DNA, essa modificação é catalisada pelas enzimas DNA metiltransferases (DNMTs), ocorrendo a adição covalente de um grupo metil ao

carbono 5’ do anel pirimídico da citosina, resultando em 5’-metilcitosina (5mC). Essas citosinas estão localizadas a 5’ de uma base guanina em um dinucleotídeo CpG na região promotora de diversos genes e contêm múltiplos sítios de ligação para fatores de transcrição (Chuang and Jones, 2007). Dessa forma, com a metilação desses sítios CpG, ocorre a inibição da ligação de fatores de transcrição e também a atração de proteínas ligantes de metil (MeCPs), que iniciam a compactação da cromatina e o silenciamento gênico (Klose and Bird, 2006). Em humanos, cerca de 60% dos genes contém uma região rica em CpG em seus promotores e, em geral, quando essa região é metilada, a expressão do gene correspondente é impedida.

Um conjunto de dados consistentes tem associado a metilação de DNA à esquizofrenia (Sharma, 2005). As primeiras evidências de que processos epigenéticos poderiam estar envolvidos com a psicose surgiram na década de 1970, em que foi observado que a metionina, precursora da SAM (S-adenosil-metionina, o doador de metil utilizado pelas DNMTs para metilar a citosina do DNA), quando administrada em altas doses por 3-4 semanas podia exacerbar os sintomas psicóticos dos pacientes com esquizofrenia (Wyatt *et al.*, 1971; Guidotti and Grayson, 2014). Um fator a ser considerado é que tanto a metilação global quanto a que ocorre em locais específicos do genoma, podem ser reguladas pela biodisponibilidade do doador de metil SAM (Tremolizzo et al., 2005), cuja biossíntese está alterada em cérebro de portadores de esquizofrenia (Guidotti et al., 2007).

A maior parte dos estudos focou na quantificação da metilação em promotores de genes candidatos, sendo que os primeiros trabalhos em tecidos cerebrais *post-mortem* focaram nos genes *RELN*, *COMT* e *SOX10* (Abdolmaleky *et al.*, 2005; Grayson *et al.*, 2005; Iwamoto *et al.*, 2005). O primeiro estudo em larga escala do epigenoma, utilizando tecido *post-mortem* obtido do córtex frontal, avaliou por *microarray* a metilação de aproximadamente 12.000 sítios CpG do genoma e encontrou diferenças significantes em diversos genes relacionados à psicose, incluindo genes envolvidos no desenvolvimento neuronal e nas vias glutamatérgicas e GABAérgicas (Mill et al., 2008).

Semelhantemente aos estudos de expressão gênica, não é possível estudar os fatores epigenéticos que atuam em uma doença apenas nos tecidos cerebrais *post-mortem.* Como as manifestações clínicas da esquizofrenia começam nos estágios prodrômicos, seguidos do PEP na adolescência e subsequente deterioração, existe uma real necessidade de utilização de tecidos periféricos (Guidotti et al., 2014). Além disso, diversos estudos em metiloma de humanos e animais indicaram uma homologia

entre cérebro e sangue (Davies *et al.*, 2012; Provencal *et al.*, 2012; Kundakovic *et al.*, 2014).

Alguns estudos revelaram uma tendência à hipometilação global em células de sangue periférico de portadores de esquizofrenia (Shimabukuro *et al.*, 2007; Melas *et al.*, 2012; Nishioka *et al.*, 2013), embora outros trabalhos não tenham identificado diferenças (Bromberg et al., 2008; Dempster et al., 2011). Também foram observadas diferenças na metilação de regiões promotoras do receptor dopaminérgico D2 em sangue periférico de gêmeos monozigóticos discordantes para a esquizofrenia (Petronis et al., 2003). Interessantemente, alguns desses achados foram concordantes entre diferentes tecidos (cérebro *post-mortem* e sangue), como para os genes *RELN* (Aberg et al., 2014) e *BDNF* (Auta et al., 2013; Ikegame et al., 2013). Paralelamente, também foi observado um aumento na expressão dos genes das enzimas DNA metiltransferases (*DNMT1*, *DNTM3a* e *DNMT3b*) em tecido cerebral e periférico (Veldic et al., 2004; Veldic et al., 2005; Zhubi et al., 2009).

Investigando a metilação de DNA em sangue periférico, Liu et al. (2013) identificaram 16 sítios CpGs hiper- ou hipometilados, sendo que a via biológica mais desregulada envolvia genes de resposta inflamatória (Liu et al., 2013). Mais recentemente e utilizando sequenciamento de nova geração, Aberg et al. (2014) identificaram 25 sítios associados com a esquizofrenia ao comparar amostras de 759 pacientes e 738 controles. Desses, o mais significantemente associado localizava-se no gene *FAM63B*, e interessantemente, um sítio localizava-se no gene *RELN* (Aberg et al., 2014). Quanto ao tratamento, foi observado que medicamentos utilizados para doenças psiquiátricas, como o ácido valpróico, podem ser capazes de promover uma hiperacetilação de histonas, outra modificação epigenética capaz de regular a expressão gênica (Phiel et al., 2001). Além disso, Melas et al. (2012) observaram uma hipometilação global em leucócitos de pacientes com esquizofrenia que era parcialmente recuperada pelo tratamento com haloperidol (Melas et al., 2012).

Um estudo combinou dados de genotipagem, expressão e metilação, encontrando 11320 CpGs diferencialmente metilados entre pacientes com esquizofrenia e controles. Desses, 1095 CpGs estavam associados com 1226 transcritos em *cis*. Comparando-se esses transcritos entre casos e controles, 391 (31,89%) estavam diferencialmente expressos. Por fim, esses dados também foram comparados com dados de genotipagem provenientes de GWAS. O resultado mais significante identificou que o SNP rs11191514 localizado no gene *CNNM2* estava

associado com a esquizofrenia e com uma metilação diferencial em um sítio próximo do gene *CALHM1* entre casos e controles. A metilação de CpGs adjacentes ao gene *CALHM1*, por sua vez, estava correlacionado com a expressão desse gene, o qual também estava diferente entre pacientes e controles (van Eijk et al., 2014). Esse estudo sugeriu que a combinação de diferentes dados (genoma, transcriptoma e metiloma) pode aumentar o poder da associação, o que não seria possível ao analisar individualmente cada dado com uma amostra pequena.

ARTIGOS CIENTÍFICOS

4 ARTIGOS CIENTÍFICOS

Como mencionado anteriormente, esta tese originou quatro artigos sendo dois já publicados. No primeiro (Santoro et al., 2015), estudamos a expressão de genes candidatos no sangue de indivíduos em EMR, pacientes em PEP e controles. Esse estudo foi o primeiro a verificar a expressão gênica no grupo EMR. Apesar do baixo tamanho amostral de indivíduos EMR (N=22) fomos capazes de identificar dois genes diferencialmente expressos, sendo que o gene *UFD1L* estava com a expressão aumentada em EMR quando comparado a controles e ao PEP, demonstrando uma alteração específica para este grupo. Por outro lado, o gene MBP estava hipoexpresso em EMR quando comparado ao PEP. A apresentação desses dados recebeu uma "Menção honrosa no XVI Congresso do Programa de Pós-graduação em Biologia Estrutural e Funcional" como melhor projeto em 2013 (Anexo 3), além de receber o prêmio "*Early Career Investigators Award - Travel Award*" of *22nd World Congress of Psychiatric Genetics* (Anexo 4).

No segundo artigo (Santoro et al., 2016) realizamos uma revisão conceitual dos últimos trabalhos de GWAS publicados na área de psiquiatria, dando especial atenção ao PRS como ferramenta para identificar o risco individual para doenças psiquiátricas. Uma das metas ao escrever esse artigo era revisar a literatura e atualizar o conhecimento para a subsequente associação do PRS com dados clínicos de pacientes em PEP (terceiro artigo submetido para JAMA Psychiatry).

Neste terceiro artigo validamos com sucesso o método de PRS de esquizofrenia na amostra brasileira, além de encontrarmos uma correlação negativa desse escore com os sintomas depressivos nos pacientes tratados com risperidona por dois meses.

No quarto e último manuscrito da tese, o qual está em preparo, nós encontramos genes diferencialmente expressos e regiões genômicas diferencialmente metiladas entre os grupos. Curiosamente, alguns genes e regiões já foram previamente associados com a esquizofrenia no maior GWAS já realizado

(Ripke et al., 2014). Uma análise de vias apontou, ainda, que a via do sistema imunológico parece estar hiper-regulada durante o PEP em comparação com controles e com o PEP tratado. Esse trabalho recebeu recentemente um prêmio "*Early Career Investigators award - Travel Award*" of *5th Biennial Schizophrenia International Research Society Conference* (Anexo 5).

O anexo 6 apresenta detalhadamente o passo-a-passo (*scripts* do R Studio e do Plink 1.9) das análises de bioinformática/estatística utilizadas nos artigos 3 e 4.

Os dois primeiros artigos foram financiados pelos projetos Fapesp 2010/08968-6, 2011/50740-5 e 2012/50316-1 os experimentos e análises foram realizados na Disciplina de Genética do Departamento de Morfologia e Genética e no LINC da UNIFESP. Os dois últimos artigos foram financiados pelos projetos 2011/50740-5 e 2014/50830-2 e todas análises genéticas foram realizadas no SGDP do King's College London durante o estágio em Pesquisa no exterior do aluno (FAPESP 2014/22223-4) sob a supervisão do Dr. Gerome Breen.

4.1 Artigo 1 – Santoro et al., 2015 (Artigo publicado na World Journal of Biological Psychiatry em Junho de 2015)

Objetivo: Este estudo teve como objetivo investigar a expressão gênica em sangue periférico em três grupos, indivíduos em estado mental de risco para psicose (EMR), pacientes em primeiro episódio psicótico (PEP) e controles saudáveis (HC).

Casuística e metodologias: Foram recrutados 22 indivíduos em EMR, 66 em PEP e 67 HC. Investigamos a expressão de 12 genes utilizando ensaios *Taqman* e utilizamos o Modelo Linear Univariado (GLM) seguido por uma correção de Bonferroni para comparações múltiplas para análise estatística.

Principais resultados: Encontramos que o gene *UFD1L* gene apresenta expressão aumentada no grupo EMR em relação ao PEP e ao HC ($P = 3{,}44\times10^{-6}$; $P = 9{,}41\times10^{-6}$). Por outro lado, os genes *MBP* (proteína básica da mielina) está com a expressão diminuída em EMR quando comparado com o PEP (P = 6,07 × 10-6). Além disso, o gene *DISC1* (*Disrupted in Schziophrenia* 1) também está aumentado em EMR se comparado ao PEP, mas perde significância estatística quando corrigido para a idade.

Conclusão: Estes genes estão diretamente relacionados com os processos de desenvolvimento neurológico e têm sido associados à esquizofrenia. Descobertas recentes descreveram que o aumento da expressão de *DISC1* pode parar ou diminuir a expressão de *MBP*, assim, pensamos que estas alterações nos indivíduos EMR poderiam estar associadas a um processo comum. Com relação ao gene *UFD1L,* ele mostrou um padrão de expressão específico para o grupo EMR, baseado na função desse gene, sugerimos que o grupo EMR está sob um estresse celular agudo, exigindo níveis elevados de Ufd1.

Gene expression analysis in blood of Ultra-High Risk subjects compared to first-episode of psychosis patients and controls

Running Head: Gene expression in blood of Ultra-High Risk subjects

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ABSTRACT:

Objective: This study aimed to investigate peripheral-blood gene expression in Ultra-High-Risk (UHR) subjects compared to first-episode psychosis individuals (FEP) and healthy controls (HC).

Methods: We enrolled 22 UHR, 66 FEP and 67 HC and investigated the expression of 12 genes using Taqman-assays. We used the Univariate General Linear Model, as well as Bonferroni correction for multiple comparisons.

Results: We found that *UFD1L* gene was upregulated in UHR group compared to HC and FEP ($p=4.13x10^{-5}$; $p = 1.1x10^{-4}$). *MBP* was downregulated in UHR compared to FEP ($p = 7.29x10^{-5}$). *DISC1* was also upregulated in UHR compared to FEP but lost statistical significance when corrected for age.

Discussion: These genes are directly related to neurodevelopmental processes and have been associated to schizophrenia. Recent findings have found that *DISC1* overexpression can disrupt *MBP* expression, thus, we believe that these alterations in UHR individuals could be associated with a common process. Curiously, *UFD1L* showed a different pattern of expression only for UHR group, based on its function, we could suggest that UHR subjects are under an acute endoplasmatic reticulum stress, demanding elevated levels of Ufd1. Further studies can improve knowledge on disease progression and putative targets to preventive strategies.

Key Words: Schizophrenia; Gene expression; *UFD1L; MBP; DISC1*.

1 INTRODUCTION

The prodromal period for schizophrenia (SCZ), operationally defined in prospective studies as a state called Ultra-High Risk (UHR), is characterized by the presence of attenuated psychotic symptoms accompanied by a cognitive and functional decline (McGlashan and Johannessen, 1996; Yung and McGorry, 1996). Transition to psychosis ranges from 18% at 6 month- to 36% after 3 year- follow up (Fusar-Poli et al., 2012).

Schizophrenia is a neurodevelopmental disorder with major contribution of genetic factors (estimated heritability around 80%) (Sullivan et al., 2003). Thus, the comparison of peripheral-blood gene expression in individuals at UHR and in the first episode of psychosis (FEP) has the potential to provide new insights about the neurobiology of the critical moment when someone transits from an at-risk state to a full-blown and potentially severe mental illness. In addition, the investigation of potential biomarkers associated with the development of psychosis may enhance the predictive power of the current UHR criteria. In this way, we selected 12 genes with known or potential relation with schizophrenia involved in neurodevelopment, drug abuse or neurotransmitter metabolism. The aim of this study was to characterize peripheral-blood gene expression of target neurotransmission genes in the early stages of psychosis, including individuals at-risk and in their first episode (UHR and FEP) and to compare them with a healthy control group (HC).

2 METHODS

We recruited 22 UHR, 66 FEP and 67 HC aged 14 to 44 years. All the subjects signed a consent prior the inclusion in the study.

UHR individuals were recruited in the "Program of Recognition and Intervention in subjects At-Risk Mental States" (PRISMA) and they were help-seeking individuals or subjects referred by primary and secondary care services. The definition of UHR was confirmed using the Comprehensive Assessment of At-Risk Mental States (CAARMS). Individuals were classified in three possible groups: a) brief and intermittent psychotic symptoms (BLIPS) b) attenuated positive symptoms (APS) and c) family history of psychosis or schizotypal personality plus impairment in functioning in the last year (HDec).

FEP patients were recruited in the "Center for Integrated Mental Health of Santa Casa de São Paulo" (CAISM) and diagnosed according to DSM-IV criteria, using SCID-I. All individuals were antipsychotic-naïve and had no previous history of: psychotic episodes due to a general medical condition, substance-induced psychotic disorder, mental retardation and psychotic episode related to bipolar disorder or depression.

HC were recruited in UNIFESP and in the "*Centro de Solidadriedade ao Trabalhador*" and evaluated in the "*Programa de Esquizofrenia da UNIFESP*". They did not meet criteria for any axis I DSM-IV mental disorder, according to SCID-I, have no family history of psychotic or mood disorders in first-degree relatives.

2.1 Blood samples preparation:

RNA samples were collected via vein puncture using PAXgene tubes for mRNA stabilization, and isolated using PAXgene blood RNA kit (Qiagen) according to the manufacturer's protocol. Quantity and quality of the extracted RNA were measured to ensure that samples were not degraded. cDNA was synthetized using the High-Capacity

cDNA reverse transcription (RT) kit (Life Technologies) with a standard RNA input of 400ng.

2.2 Quantitative PCR:

We investigated the expression of 12 candidates genes (*AKT1, COMT, DGCR2, DGCR8, DICER1, DISC1, DROSHA, MBP, NDEL1, PAFAH1B1, TNF* and *UFD1L*), two endogenous genes (*ACTB* and *GAPDH*) using Taqman Low Density Array (TLDA) technology (Life Technologies), which is preconfigured in a 384-well format and spotted on a microfluidic card in duplicate assays. We selected 12 genes based on Gene Cards website information regarding gene expression in blood and the relevance of each gene in different nervous system functions and the relevance to schizophrenia. We then validated if expression was detectable by TLDA.

We followed the manufacturer's protocol for all steps using standard TaqMan Universal Master Mix without UNG and ViiA™ 7 Real-Time PCR System (Life Technologies).

2.3 Statistical Analysis:

Using the Comparative Crt method, geometric mean of the endogenous genes was calculated in order to obtain the ΔCrt values for each gene of interest. We performed a Univariate General Linear Model to verify gene expression differences comparing UHR group with FEP and HC groups. We used Bonferroni test for Post Hoc and correction of multiple comparisons.

We used Pearson correlation test to verify if the genes differentially expressed could be involved in a common or convergent processes. We considered significant p values < 0.05.

3 RESULTS

Clinical and demographic variables are shown in Table 1. Mean age of UHR was 18.3 years and, as expected, significant lower than FEP mean age of 25.9 years ($p<0.001$). There were no gender differences among all groups. Among the 22 individuals in UHR group, there were 14 fulfilling criteria for APS, 4 fulfilling criteria for BLIPS and 4 for HDec.

As we found a significant difference in age between groups, we tested if this variable could influence our significant results using it as covariate in the GLM test. We found that *UFD1L* gene was upregulated in UHR group compared to HC and FEP, and that *MBP* was downregulated in UHR compared to FEP. *DISC1* was also upregulated in UHR compared to FEP, but after including age as a covariate, it lost the statistical significance. Table 2 resumes our significant findings.

4 DISCUSSION

We found three genes differentially expressed in the analysis ofUHR subjects when compared to HC and FEP patients. These three genes are known for their specific functions in processes related to neurodevelopment.

MBP is critical for myelin membrane biogenesis, entry regulation of proteins into membrane sheets (Boggs, 2006), and is the major constituent of the myelin sheath of oligodendrocytes and Schwann cells. It is suggested that defects in myelin insulation may lead to reduced nerve impulse propagation, and consequently, compromise of neuronal and glial functions (Martins-de-Souza, 2011). Moreover, MBP is a known marker for neurodegenerative diseases, such as multiple sclerosis, demonstrating its close relation to neurodevelopment (Lieberman, 1999). Conversely, dysfunction of oligodendrocytes has been considered a pivotal feature of SCZ pathogenesis, mainly due to its impact on brain connectivity (Davis et al., 2003; Kubicki et al., 2005).

Intriguingly, Hattori et al. 2014 described that *DISC1* overexpression in *in vitro* neurons disrupts not only induction of *MBP* expression, but also transformation of oligodendrocytes to a complex morphology (Hattori et al., 2014). In the same way, we observed an upregulation of *DISC1* that can be disrupting *MBP* expression, suggesting that these alterations may reflect a common process, or convergent functional sequelae. However, we must highlight that *DISC1* upregulation lost statistical significance after age correction, indicating that its expression could be influenced only by age.

Supporting these disturbances in myelination of SCZ, transcriptome and proteome studies found *MBP* differentially expressed in several brain regions of patients, however, most of them found a decreased expression of *MBP* in patients (Martins-de-Souza, 2010; Martins-de-Souza et al., 2010; Matthews et al., 2012; Parlapani et al., 2009). In this study, we found a dowregulation of *MBP* expression in UHR compared to FEP, however, one might say that *MBP* is actually upregulated in FEP compared to UHR. Indeed, its expression

is also upregulated in FEP compared to controls, although we did not find statistical significance (p= ; Fold Change=). Similarly, other studies suggested that antipsychotics could dowregulate *MBP* expression (REFS). Similarly to our results, Kumarasinghe et al. (2013) reported an upregulation of *MBP* in a sample of antipsychotic-naive schizophrenia patients compared to controls.

In this way, we propose that this upregulation of *MBP* in FEPs blood can be a key turning point in the disorder and that the downregulation observed previously in SCZ patients might be a treatment effect. However, new longitudinal studies should be conducted to understand if UHR individuals presents an upregulation of *MBP* during the transition to psychosis and also if *MBP* expression decreases in FEP after chronic antipsychotic treatment.

Ubiquitination is a post-translational modification that plays a central role in regulating protein half-life (Myung et al., 2001). In eukaryotes, post-translational conjugation to ubiquitin is an obligatory preliminary step for degradation of many proteins (Ciechanover, 1994). *UFD1L* gene encodes the human homolog of yeast ubiquitin-fusion-degradation 1 protein and is located in 22q11.2 region. In humans its expression begins from 10 weeks of gestational age and continues to be transcribed throughout fetal and post natal life (Novelli et al., 1998), thus, it was suggested that this gene was involved in neurodevelopment.

Ufd1 acts together with Npl4 as an adaptor protein, which recognizes mono and poli-ubiquitinated proteins (Park et al., 2005) for p97 specific activity (AAA-ATPase). This p97-Ufd1-Npl4 complex acts in endoplasmatic reticulum (ER) associated degradation (ERAD), extracting misfolded proteins marked with ubiquitin from ER to be degradated in cytosol by the proteasome (Ye et al., 2001). Thus, it was suggested that Ufd1 is directly associated in the stress response in ERAD (Chen et al., 2011).

UFD1L SNP, rs5992403, has been associated with schizophrenia (De Luca et al., 2001), with early-onset of the disorder (Ota et al., 2010) and with a deficit in the set-shifting task (which represents a deficit in fundamental dimensions of cognitive in schizophrenia) (Ota et al., 2013). Moreover, its genomic region (22q11.2) is deleted in DiGeorge syndrome, which is considered one of the main genetic risks, beside monozygotic twins, for schizophrenia (~25% displays psychotic symptoms in early adulthood) (Bassett et al., 2005). Likewise, deletions in 22q11.2 are more common in schizophrenia subjects than in the general population (Christofolini et al., 2011).

In this study, we found a significant upregulation of *UFD1L* in blood of UHR subjects when compared to HC and to FEP patients. This result suggests that this upregulation is specific for the UHR group. Moreover, upregulation in *UFD1L* had a high predictive power and a moderate effect size (table 2), indicating a good potential to be a future biomarker specific for the UHR group.

Accordingly to UFD1L function, we could suggest that UHR subjects are under an acute ER stress, demanding elevated levels of UFD1. In this way, future studies should

focus in the ERAD system and investigate the differences between individuals before and after transition to psychosis.

The results of this study should be interpreted at light of some limitations. One of them is the sample size, especially in UHR group. It is a consequence of the difficulty in identifying early signs of psychosis in adolescents and young adults and properly refer them. Although our main aim was to find biomarkers to identify UHR group, it would be very valuable to understand how these alterations in blood could influence the brain tissue, which is not possible in humans. Another point to consider is that UHR subjects are being followed for a short period of time (mean follow-up period = 1 year) and only one patient transited to psychosis (not included in the FEP group). We used standardized criteria and instrument (CAARMS) and so far, our transition rate is similar to what was recently reported.

Accumulating evidence indicates that SCZ involves subtle cytoarchitectural abnormalities that arise during neurodevelopment. Nevertheless, the underlying molecular mechanisms are still unclear. In this study we found three genes differentially expressed in blood of UHR group compared to HC (*UFD1L*) and FEP (*UFD1L* and *MBP*). These genes have been previously studied in SCZ and are considered to be directly related to neurodevelopment. Moreover, these genes displayed a great potential as biomarkers, which in the future could enhance the identification of UHR subjects. Here, we highlighted *UFD1L*, which showed a different pattern of expression in UHR group compared to HC and FEP. Concerning *MBP* and *DISC1*, they seem to be involved in a common process, however new studies should be conducted to understand this mechanism.

5 Conflict of interest and Funding

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Table 1. Description of UHR, FEP and HC groups concerning gender, mean age, antipsychotic use and subgroup diagnosis.

Group	N	Mean age (SD)		Sex	Antipsychotic-naïve	Diagnosis
UHR	22	18.3	(3.52)	14M; 8F	17/22	4BLIPS; 15APS; 3HDec
FEP	66	25.9	(7.44)	39M; 27F	66/66	First episode of schizophrenia confirmed after 8weeks of treatment
HC	67	26.3	(8.07)	40M; 27F	67/67	No familial history of psychosis

SD: Standard deviation; UHR: Ultra High Risk for psychosis subjects; FEP: First episode of psychosis patient; HC: Healthy control; BLIPS: Brief Limited Intermittent Psychotic Symptoms; HDec: Familial history of psychosis + functional decline.

Table 2. Significant findings after Bonferroni correction for multiple comparisons.

GENE	N	Comparison	Adjusted p-value	Fold Change	Effect size	Observed power
UFD1L	HC=66 UHR=22 FEP=66	UHR/HC	$4.1x10^{-5}$	1.42	0.16	0.99
		UHR/FEP	$1.1x10^{-4}$	1.42		
MBP	UHR=22 FEP=65	UHR/FEP	$7.29x10^{-5}$	0.49	0.15	0.99
DISC1	UHR=22 FEP=64	UHR/FEP	0.015*		1.47	0.09

*** *DISC1* lost statistical significance after using age as covariate**

4.2 **Artigo 2 – Santoro et al., 2016 (Artigo publicado na *American Journal of Medical Genetics part B* em Julho de 2016)**

Resumo:

Na última década, numerosos avanços foram atingidos na área da genética psiquiátrica. Particularmente, os estudos de associação de genética em larga escala (GWAS) têm contribuído para a descoberta de novos genes e vias associadas aos transtornos psiquiátricos.

Ao mesmo tempo, com o aumento do tamanho amostral destes GWASs graças aos consórcios internacionais, o escore poligênico de risco (PRS) se tornou uma importante ferramenta para a identificação e avaliação do risco genético individual para determinado fenótipo.

Esta revisão apresenta os últimos resultados GWAS para cada doença psiquiátrica e foca na importância do PRS.

A current snapshot of common genomic variants contribution in psychiatric disorders

Running head title: Genomic variants in psychiatric disorders

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Key words: GWAS, psychiatry, polygenic risk score, psychiatric disorder.

Abstract

In the past decade, numerous advances were achieved in psychiatric genetics. Particularly, the genome wide association studies (GWAS) have contributed to uncovering new genes and pathways associated to psychiatric disorders (PDs). At the same time, with increasing sample sizes in the GWAS, the polygenic risk score (PRS) promoted an additional tool for identification and evaluation the genetic risk quantitatively in PDs. This concept review presents the state of the art GWAS analysis and PRS focusing on the genetic underpinnings of PDs.

5 Introduction

Psychiatric disorders (PDs) are leading causes of disease burden worldwide, with important impact at individual, family, and society levels, representing a high cost for the health system (Campion et al., 2012; WHO, 2008). The best current therapeutic strategies treating mental disorder patients reduce the disease manifestation by only 28% (Andrews et al., 2004). In parallel, research in neuroscience has shown that the most severe PDs are of neurodevelopmental basis resulting from a dynamic phase-specific interplay of biological and environmental factors that lead to disruption in normal brain assembly (Stachowiak et al., 2013). This perspective partially explains why the current employed symptoms based on treatment is usually not enough for a full functional recovery and emphasizes the urge for preventive measures that can avoid or attenuate pathological processes onset and/or progression.

PDs are highly heritable, twin and family studies have shown a heritability around 0.9 for autism, 0.8 for schizophrenia, and 0.6-0.8 for bipolar disorder (Craddock and Sklar, 2009; Lichtenstein et al., 2009; McGuffin et al., 2003; Sullivan et al., 2003), although identifying their genetic basis is still challenging.

The advance of the Genome Wide Association Studies (GWAS), which identify hundreds of thousands of single nucleotide polymorphisms (SNPs) and other types of genetic variations, has been particularly successful in uncovering new genes and pathways of diseases. The studies revealed many targets for hypertension and diabetes for example, opening opportunities for new treatments and diagnostic approaches (Manolio, 2010). On the other hand, for PDs, it became evident that no single variant has a large influence on risk, but rather thousands of variants confer small effects to PDs risk (Glessner et al., 2010b; Klein et al., 2010; McClellan and King, 2010).

GWAS results are robust and once conducted under standard quality control measures, they essentially replicate across different platforms. However, GWAS require a large sample size for optimal statistical power, mainly to identify common variants with small effects. In the last few years, the Psychiatric Genomics Consortium (PGC) has been overcoming this issue, successfully reaching large sample sizes in different PDs (Cross-Disorder Group of the Psychiatric Genomics, 2013; Major Depressive Disorder Working

Group of the Psychiatric et al., 2013; Neale et al., 2010b; Psychiatric, 2011; Schizophrenia Working Group of the Psychiatric Genomics, 2014).

Two major hypotheses direct genetics investigation in complex disorders: the common variant common disease (CVCD) and rare variant common disease (RVCD) models. The CVCD model proposes that the genetic risk in an individual (or population) is attributable to many high-frequency variants, each conferring modest level of risk (Risch and Merikangas, 1996). In opposition, the RVCD speculates that the genetic risk in an individual can be explained by rare mutations conferring high risk and, as a consequence, the common disease might reflect a large number (hundreds or thousands) of different causes of low frequencies (typically less than 1 out of 1,000 individuals), but with large proportion of aggregated risk (Bodmer and Bonilla, 2008)

In this article, we aim to lead a concept review about the current advances brought by GWAS data, concerning mainly the CVCD model, and thus, the common variants (SNPs), for the major PDs and how these data may be leveraged to develop potential opportunities for diagnosis measures and treatments. References for this review were selected using Pubmed. Searches were performed using the term "genome wide association study" with: "mental disorders", "psychiatric disorders", "schizophrenia", "bipolar disorder", "anxiety", "major depression", "obsessive compulsive disorder", "posttraumatic stress disorder" and "Attention-deficit/hyperactivity disorder". We considered articles published since 2009, until June 2016, emphasizing the newest and larger sample size GWAS.

5.1 **Current Advances**

Over a hundred GWAS focused on PDs were performed hitherto, most on Alzheimer's disease (AD), bipolar disorder (BD), and schizophrenia (SCZ), along with the efforts in attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), major depressive disorder (MDD), obsessive compulsive disorder (OCD), and drug abuse related disorders (Glessner et al., 2010a; Smoller, 2013; Stewart et al., 2013; Sullivan, 2010). Collectively, these studies yielded several advances in the genetics knowledge on PDs. For instance, several rare copy number variations (CNVs) have been reported for

ADHD, SCZ and ASDs (Glessner et al., 2010a; Glessner et al., 2009). On the other hand, hundreds of new common *loci* have been implicated for each PD (Major Depressive Disorder Working Group of the Psychiatric et al., 2013; Neale et al., 2010b; Psychiatric, 2011; Schizophrenia Working Group of the Psychiatric Genomics, 2014) or among them (Cross-Disorder Group of the Psychiatric Genomics, 2013). For now, the best achievements were made in SCZ and ASD.

Differently from the other major PDs, Autism affects individuals during the early childhood stages and thus, seems to be more related to the RVCD model. El-Fishawy & State 2010, argued that it is probably related to a natural selection, based on the reproductive fitness of Autism. Furthermore, Autism has shown the lowest fecundity among PDs (El-Fishawy and State, 2010; Power et al., 2013). In the last years, many CNVs were associated to ASD, however the specific mechanism is still unclear (Sanders et al., 2015).

Although the RVCD model has been suggested for SCZ as well, the last findings suggest that the CVCD model comprise the most cases of SCZ. Over the last years, dozens of GWAS with hundreds of variants associated with SCZ were published (for a review, see (Chen et al., 2015). In addition to the regions constantly associated in GWAS, the PGC group published 83 new regions associated with the risk of SCZ, a big analysis that included over 36,989 patients and 113,075 healthy controls (Schizophrenia Working Group of the Psychiatric Genomics, 2014). It is even estimated that a new common variant can be identified for each 250 cases and 250 controls added to the sample size.

Recently, a mega-analysis using genome-wide genotyping data identified specific variants underlying genetic effects shared among five PDs (a cross-disorder study) in the PGC (Cross-Disorder Group of the Psychiatric Genomics et al., 2013; Smoller, 2013). Applying univariate and bivariate methods for the estimation of genetic variation within, and the covariation between disorders and SNPs, allowed the investigators to explain about 17-29% of the variance observed. Taken together, the empirical evidence of a shared molecular signature encourages the investigation of a potential common pathophysiology for PDs, highlighting the fundamental importance of integrative collaboration to optimize the study power for new findings.

Among these PDs, BD and SCZ are the ones with higher shared heritability (Lichtenstein et al., 2009). Despite the smaller sample size in comparison to the SCZ GWAS, many regions have been associated to BD, some of them unique to the disorder and independent from those shared with schizophrenia.

Although this shared heritability makes sense in the light of PDs with a common process, it also points to a possible weakness of these studies. The confoundable diagnoses and the overlap among the PDs, especially within the spectrum of particular phenotypes, reinforces the need to discuss the boundaries among the psychiatric diagnosis.

It is important to note that despite the advances for SCZ, ASD, and BD, the GWAS have not been able to aid in the understanding of the genetic aspects of MDD (Major Depressive Disorder Working Group of the Psychiatric et al., 2013). This could mean that MDD is even more complex and might be associated more with environmental factors and/or the response of the individual to adverse environments, involving other genetic features such as gene expression or epigenetic modifications during its onset. Noticeably, a large GWAS mega-analysis (approximately 20,000 participants) for MDD did not identify significant findings (Major Depressive Disorder Working Group of the Psychiatric et al., 2013).

There are few published data for other PDs such as anorexia nervosa (AN), OCD, ADHD and post-traumatic stress disorder, and thus, the role of common and rare variation remains unknown (Collins and Sullivan, 2013; Hinney et al., 2011; Levy et al., 2012; Neale et al., 2010a; Wang et al., 2011). Probably, this lack of conclusive information is more related to the smaller sample sizes than of what is required to identify robust loci and replicable findings, which are expected to be overcome by the consortiums in the next few years.

Concerning the Anxiety Disorders, most GWA studies focused on individual anxiety phenotypes such as panic disorder, phobias or generalized anxiety and found a few regions associated to the phenotype. Recently, Otowa et al., investigated a shared anxiety disorder susceptibility performing a meta-analysis with different anxiety disorders cohorts and testing an association with a quantitative trait of anxiety beside the classic case-control association (Otowa et al., 2016). In this study, beside the identification of novel

susceptibility loci shared among anxiety disorders, they found that the quantitative phenotypic factor scores analysis was able to identify large number of associated SNPs than the classic case-control method (Otowa et al., 2016). Similarly, many studies have demonstrated the advantages of associate the genotype to single traits or PD dimensions, rather than specific DSM-V specific disorders. As an example, Terraciano et al 2010 found that many loci might influence personality traits (Terracciano et al., 2010), equivalently, Smith et al investigated the neuroticism trait in a sample over 106K individuals and identified 9 loci associated to the trait (Smith et al., 2016).

Polygenic Risk Scores

Purcell et al. (2009) describe the use of a polygenic score. This study demonstrated common points between SCZ and BD, and that this sharing was high, not only for the significant variants in the array, but also for those that were not significant. This was most probably due to a sample size problem, taking into account the premise that PDs are polygenic, and variants that are active in them have very low effect size (Purcell et al., 2009).

With the exponential increase in the sample size of the international consortia, this tool becomes even more useful. With a GWAS as a base, it is possible to calculate in another independent sample the genetic score of each individual to that disease. The PRS uses a threshold value of p (and not the regularly conservative GWAS significance threshold of $p<5\times10^{-8}$) of the variants in the base sample to select which enter the analysis. Then, from the effect size (or odds ratio) in the base sample and the number of each variant risk that the individual has in the target sample, a value is calculated for each variant and added, so that the individual in the target sample has a score of the sum of the risk of each variant (Dudbridge, 2013; Purcell et al., 2009).

One advantage is that the PRS does not need a giant sample size for the target sample as long as it is estimated from a very large sample (Dudbridge, 2013). With this score we can either verify the consistency between the base and the target sample (for example, to the same disease) as well as use this score as a variable to correlate with other

clinical traits. The potential of PRS is very broad, and in the coming years it might become a fundamental step for any study involving genetic risk.

The PRS has presented some interesting results, such as the association among the top five PDs (2013; Demirkan et al., 2011). For SCZ, the PRS has been correlated with quantitative variables, such as severity of symptoms (Derks et al., 2012) and prefrontal activity (Walton et al., 2013). For BD, PRS was correlated to function and brain structures in individuals at risk (Whalley et al., 2012; Whalley et al., 2013), and for depression, it was correlated with the reduction of the cortical volume in specific regions (Holmes et al., 2012). Table 1 lists the most recent and open source GWAS results that can be used to generate the PRS.

5.2

5.3 **Contribution of Genetic Variations to the Treatment of PDs**

The delay in establishing a treatment for PDs is one of the main reasons for the economic impact on PDs being so high. For example, the US spending on mental illness reached 83.6 billion in 2012 (Cohen, 2012). The genetic information could aid clinicians to act quickly and predict the response to certain drugs (Evans and McLeod, 2003). Due to individual variation to psychiatric drugs response, clinicians usually adopt a trial and error approach (Evans and McLeod, 2003; Mrazek, 2010).

Pharmacogenomics research contributes to optimize treatment for each patient through clinical application of genetic information. Several psychiatric pharmacogenomic tests were recently developed, including tests for the determination of metabolic status, risk of agranulocytosis and metabolic syndrome, and selection of beneficial medications (Arranz and Kapur, 2008). Pharmacogenomic tests could result in better clinical outcomes for patients with appropriate dosage regimens for poor metabolizers (PMs) and ultra-rapid metabolizers (UMs), potentially reducing the time to drug response and functional impairment, and thus, promoting a faster recovery. This information is essential to personalized medicine. Now, clinicians are able to predict the drug response or adverse effects, adjusting medication dosage, or even choosing another drug before the treatment (Gardner et al., 2014).

In the last decade, many studies have found association between genetic variations and psychiatric drug responses or adverse effects. Polymorphic drug-metabolizing enzymes are responsible for the metabolism of the majority of psychotropic drugs. Cytochrome P450 (CYP450) are the major family of enzymes mediating the phase I of drug metabolism (Stingl et al., 2013). CYP2D6 only represents 1-5% of the CYP liver content, but is responsible for the oxidative metabolism of up to 25% of commonly prescribed drugs such as antidepressants, antipsychotics, opioids, antiarrhythmics, and tamoxifen (Samer et al., 2013). These effects are especially important to psychiatry treatment in which such polymorphisms were found relevant for 14 out of 36 antidepressants, requiring at least double the drug dose in extensive metabolizers (EMs) in comparison to PMs, and for one third of the assessed antipsychotics (Kirchheiner et al., 2004). Regarding antipsychotics, several reports show that CYP2D6 drugs have higher risks to develop tardive dyskinesia and other parkinsonian side effects (Brockmoller et al., 2002).

Three GWAS of antidepressant response have been reported (Garriock et al., 2010; Ising et al., 2009) without having any major contribution to effective treatment. Regarding BD, the standard treatment employs mood stabilizers, namely lithium, and anticonvulsants (valproate, carbamazepine, and lamotrigine). The mechanisms of mood stabilizers are not completely known. Several groups have been studying usual candidate genes, i.e. MAOA, COMT, 5HTT, TPH1, BDNF (Duffy et al., 2000; Serretti et al., 1999a; Serretti et al., 1999b; Serretti et al., 2000) although evidence that the role of any variation of these genes in the treatment response to mood stabilizers remains inconclusive.

In the psychosis field, a few GWAS of antipsychotic treatment responses have been reported (Alkelai et al., 2009; Lavedan et al., 2009; Volpi et al., 2009). Three of these studies were performed from the Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) trial in which patients with SCZ were randomized to treatment with either a second generation of antipsychotics (olanzapine, quetiapine, risperidone, or ziprasidone) or a first generation of antipsychotics (e.g. perphenazine) (Zandi and Judy, 2010). The remaining two GWAS (Ventimiglia et al., 2010) came from a phase three randomized trial of iloperidone, an investigational new drug for the treatment of SCZ (Vanda

Pharmaceuticals). The most reliable finding was in the NPAS3 gene, previously associated to SCZ (Lavedan et al., 2009; Volpi et al., 2009).

In 2011, genetic variants were reported that clustered on certain gene networks involving metabotropic glutamate receptors (mGluR) that predispose to ADHD (Elia et al., 2011). For patients harboring mutations implicating mGluR hypofunction, mGluR agonists and positive allosteric modulators that activate mGluR signaling can potentially restore normal neurophysiology. This discovery lead to the development of an mGluR agonist drug which can be the first personalized therapeutic developed for PDs and could potentially become the first drug belonging to the class of mGluR agonists to reach the market.

Based on these examples, genomic tools can assist in the development of new drugs as well as predict adverse effects and responses to certain drugs, thereby decreasing costs to establish and stabilize the treatment.

Limitations

Despite the benefits of GWAS, these studies still have limitations that should be overcome in the future. There are controversial data regarding the findings of susceptible genes for PDs, which do not replicate consistently, showing statistically significant effects in some studies but not in others. While this may simply reflect the difficulty of measuring small effects, which can require thousands of subjects to reach significance and power, differences in the genetic background of experimental and control groups can also lead to false-negative or false-positive results (Ward and Kellis, 2012). Avoiding such artifacts requires proper matching groups, in particular with respect to the ethnic background of the study populations.

Although GWAS present cumulative predictive power for specific phenotypes, a great portion of discoveries involve noncoding loci located relatively far from coding genes, which makes further functional experiments difficult. Prospective studies are needed to check the real power to improve early identification. One major issue is the possibility of false positives and the burden that stigma can cause to unaffected subjects erroneously diagnosed. The potential of genomics to improve detection is clear, however, this kind of

balance needs to be carefully approached. At this moment, it is evident that there is a need to improve the accuracy and the phenotype information explained by current GWAS data.

Conclusion

GWAS have been highly successful in advancing the field of genetics of PDs, by unraveling both new genes and molecular pathways involved in the pathogenesis of these disorders, although genetic mechanisms remain unknown in most instances. In addition, GWAS complements with other approaches such as CNV, micro RNAs, epigenetics, and proteomics, which are fundamental to better elucidate the underling mechanisms. GWAS has mostly contributed to the understanding of the PDs etiology and to new treatment approaches. Although still far from the prevention of psychiatric disorders, the PRS is certainly a new step towards personalized medicine and prevention. Future genetic studies allowing for more effective integration of genomic data may reduce the gap between the current body of knowledge on genetics and clinical application.

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Table 1. Open source results available to generate the polygenic Risk Scores for psychiatric disorders and other traits.

Psychiatric Disorder	N sample (case/control)	Consortia (reference)	Significant GWAS regions ($p<5x10^{-8}$)	Top candidate gene
SCZ	36,989/113,075	PGC (2014)	108	Dopamine receptor genes and other mult
BD	11,974/51,792	PGC (2011)	2	Calcium Voltage-Ga *(CACNA1C)* and T (*ODZ4)*
MDD	9,238/8,039 5,303/5,337 (women only)	PGC (Ripke et al., 2013) CONVERGE (2015)	0 2	-- Sirtuin 1 *(SIRT1)* a Inorganic Pyrophospl
ADHD	896/2,455	PGC (Neale et al., 2010a)	0	
Cross-Disorder (SCZ; BD; MDD; Autism; ADHD)	32,332/27,888	PGC (2013)	2	*CACNA1C* and Ca Beta 2 Subunit (*CAC*
OCD/TS	2,723/5,667	(Yu et al., 2015)	0	--
PTSD	NA	NA	NA	NA
Anorexia nervosa	5,551/21,080	PGC (Boraska et al., 2014)	0	SOX2 overlapping tr Family With Sequenc
Anxiety disorder	7,016/14,745 18,000 (qtrait)	ANGST (Otowa et al., 2016)	1 1	rs1709393 (non-codi Calmodulin-Lysine N-
Alzheimer	17,008/37,154	IGAP (Lambert et al., 2013)	20	Apolipoprotein E *(A* regions; 11 new asso
Neuroticism	63,661 (qtrait)	GPC (de Moor et al., 2015)	1	Membrane Associate
Educational Attainment	293,723 (qtrait)	(Okbay et al., 2016)	74	Multiple regions regu

NA= data not available; SCZ=Schizophrenia; BD=Bipolar Disorder; OCD/TS= Obsessive Compulsive Disorder and Tourette Syndrome; PTSD= Posttraumatic stress disorder; MDD= Major Depression Disorder; PGC=Psychiatric Genomics Consortium; GPC=The Genetics of Personality Consortium; ANGST= Anxiety Neuro Genetics Study; IGAP=International Genomics of Alzheimer´s Project; CONVERGE=**C**hina, **O**xford a**n**d **V**irginia Commonwealth University **E**xperimental **R**esearch on **G**enetic **E**pidemiology.

5.4 **Artigo 3 – Santoro et al., (Submetido para JAMA Psychiatry)**

Objetivo: Verificar (1) se escore poligênico de risco para esquizofrenia (PRS) é capaz de diferenciar casos e controles em uma coorte brasileira longitudinal de pacientes em primeiro episódio psicóticos (PEP) e (2) se variáveis clínicas de gravidade e de resposta à antipsicóticos se correlacionam com o PRS durante a fase de PEP e/ou dois meses após o tratamento com risperidona.

Casuística e metodologias: Foi realizada a avaliação clínica de 60 pacientes PEP virgens de tratamento com antipsicótico e após o seguimento por dois meses (9,03 ± 2,76), além de 60 controles saudáveis. Após a coleta de sangue e extração de DNA, as amostras foram genotipadas usando o *PsychArray* (*microarray* genômico desenvolvido pelo consórcio internacional de genética psiquiátrica). Foi realizada a imputação genômica dos dados utilizando o site http://imputationserver.sanger.ac.uk, para gerar a pontuação do PRS e avaliar o melhor limiar de p para a nossa amostra nós utilizamos o programa *PRSice*. Como GWAS base, foram utilizados os últimos dados disponíveis do PGC.

Principais resultados e Discussão: O PRS foi capaz de identificar casos e controles na amostra brasileira de PEP, mas com uma variância explicada moderada (19%) e com um melhor limiar de p abaixo do encontrado no PGC ($p<0.0112$). Observamos três correlações positivas que só são observadas durante o PEP entre o PRS e as variáveis clínicas, sintomas depressivos (CDSS), sintomas excitatórios (PANSS-excitatória – 5 fatores) e funcionamento (-GAF). No entanto, após dois meses de tratamento com risperidona, observamos apenas uma correlação negativa do PRS com a dimensão de depressão da PANSS e com os escores totais da CDSS. Esses resultados demonstram a importância em se trabalhar com indivíduos em PEP antes da intervenção antipsicóticos, de tal forma que após um curto período de tratamento a relação PRS e variáveis clínicas é provavelmente influenciado pelo antipsicótico que o indivíduos recebe e não com a doença propriamente dita.

Polygenic risk scores associations with pre-treatment symptoms in a longitudinal cohort of antipsychotic-naive first episode psychosis patients.

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Key words: schizophrenia, polygenic risk score, first episode psychosis, PANSS, severity.

Abstract

INTRODUCTION: Schizophrenia (SCZ) is a complex disorder with high heritability. Polygenic Risk Scores (PRS) allow access to the highly polygenic architecture of risk in schizophrenia and cumulatively sum the low effect sizes of common risk variants. To our knowledge, no previous study has been conducted on PRS in antipsychotic-naïve first episode of psychosis patients (FEP).

AIM: In this study, we aimed to (1) confirm if a schizophrenia PRS was able to differentiate cases and controls in Brazilian first episode of psychosis (FEP) patients; (2) test if the PRS was correlated to clinical symptoms and variables indicating severity and response to antipsychotics during, two months after initiation of risperidone treatment (FEP-2M).

Methods: We performed a detailed clinical assessment of 60 antipsychotic naïve patients in their FEP and after two months of Risperidone treatment. After blood collection and DNA isolation, the samples were genotyped using the Illumina PsychArray Chip and then imputed. To calculate polygenic risk scores (PRS) we used the latest available GWAS summary statistics from the Psychiatric Genomics Consortium wave 2 SCZ group as a training set. We used Poisson Regression to test association between PRS and clinical measurements correcting for the four principal components (genotyping). We considered as significant a p-value < 0.05 after Bonferroni correction.

Results and discussion: We verified that the schizophrenia PRS is relevant to the Brazilian population, with a similar variance explained (~0.15) to that observed in Northern European populations. At baseline, we found that PRS is positively associated with PANSS-excitement factor, depression (CDSS) and (-)GAF, meaning that the higher the PRS the more severe the symptomatology or impaired functioning. After treatment, these results disappear and depressive symptoms (CDSS and PANSS-depressive factor) become negatively associated to PRS. These results highlight the importance to study the FEP before the increment of antipsychotics in order to understand the true relation between genotypic and phenotypic features.

Introduction

Schizophrenia (SCZ) is a severe mental disorder that affects approximately 1% of the general population and is characterized by the presence of psychosis and other heterogeneous features, such as negative symptoms (i.e., flattened affect and social withdrawal) and disorganization symptoms (e. g. impaired cognitive function, disorganized speech and behavior). The period just after the onset of the disorder is believed to be particularly critical for prognosis because symptomatic and psychosocial deterioration progress rapidly during this phase (Birchwood et al., 1998). Moreover, reports indicate that brain abnormalities and cognitive deficits are already present (Birchwood et al., 1998), even though patients are not affected yet by factors related to disease progression, such as duration of illness and long exposure to antipsychotics (Demjaha et al., 2012; Fuste et al., 2013).

Genetics appears to play a pivotal role in SCZ vulnerability, as it has a high heritability estimates (approximately 80%) (Sullivan et al., 2003). Even clinical diversity may be skewed by individual genetic and environmental variation, twin studies reported heritability of psychotic experiences that ranged from 33 to 57% (Lin et al., 2007; Ericson et al., 2011; Hur et al., 2012). The most recent Genome Wide Association Study (GWAS) for SCZ in the Psychiatric Genomics Consortium (PGC) wave 2 (PGC2), which test the association of hundreds of thousands of single nucleotide polymorphisms (SNPs) and other types of genetic variations, has been particularly successful in uncovering new genes and pathways for the disorder ((Schizophrenia Working Group of the Psychiatric Genomics, 2014); for a review see (Santoro et al., 2016)). For SCZ (and other psychiatric disorders), it is not well accepted that, while no single variant accounts for a large proportion of cases, thousands of genetic variants act together to confer the majority of the genetic risk for the disorder - a polygenic architecture of risk (Glessner et al., 2010; Klein et al., 2010; McClellan and King, 2010).

Purcell et al. (2009) developed a method to calculate a polygenic risk score (PRS). This study demonstrated common variants between SCZ and bipolar disorder with high sharing not only for the genome wide significant variants in the array, but also for those with nominally significant p-values. With the exponential increase in the sample size of the international consortia, this tool has become even more reliable. With the PGC2 SCZ GWAS as a training sample, it is possible to calculate in another independent sample the genetic score of each individual to that disease.

The PRS uses a nominal p-value threshold to select from a base sample which variants enter the analysis. Then, using the effect size in the base sample and the number of risk variants that each individual has in the target sample, a value is calculated for each individual and summed across the genome to yield a PRS for an individual (Purcell et al., 2009; Dudbridge, 2013).

One advantage is that the PRS has a much larger effect size than any single genetic variant and does not need a large sample size for the target sample as long as it is estimated from a very large training sample (Dudbridge, 2013). With this score, we can either verify the consistency between the base and the target sample (for example, to the same disorder) as well as use this score as a variable to correlate with other clinical traits. For SCZ, the PRS has been correlated with quantitative variables, such as severity of symptoms (Derks et al., 2012) and prefrontal activity (Walton et al., 2013). For bipolar disorder, PRS has been correlated to function and brain structures in individuals at risk (Whalley et al., 2012; Whalley et al., 2013), and for depression, it has been correlated with the reduction of the cortical volume in specific regions (Holmes et al., 2012). A recent paper by Vassos and colleagues, reported that SCZ PRS is associated with diagnostic outcome in FEP patients (in a mixed sample of patients who went on to develop SCZ or affective disorders or who recovered). One recent study, reported a positive correlation between SCZ PRS and negative symptoms in an adolescent population cohort (Jones et al., 2016).

In this study we aimed to verify (1) if PRS was able to differentiate cases and controls and (2) if PRS can be correlated to variables of symptomatology, severity and response to antipsychotics during FEP stage and if this correlation is consistent during the development of the disease. To our knowledge, this is the first study to evaluate the association of PRS with baseline pre-treatment symptoms and clinical variables indicating severity and drug response in a longitudinal sample of antipsychotic naïve FEP patients.

Methods

Cohort description:

The Research Ethics Committee of UNIFESP (Universidade Federal de Sao Paulo) approved the research protocol, and all participants and family members provided written informed consent prior to enrolment in the study (CEP 0603/10 and CAAE 33148114.6.0000.5505).

Longitudinal cohort of first episode of psychosis patients (FEP):

Our cohort of antipsychotic-naïve FEP patients includes 154 subjects which were recruited from a psychiatric emergency unit in São Paulo (Brazil). The diagnosis of a psychotic disorder was established by trained psychiatrists based on the criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV), using the Structured Clinical Interview of the DSM-IV (SCID-I). Inclusion criteria were age between 16 and 40 years and no previous history of antipsychotic medication. Prior or current treatment with benzodiazepines was allowed. Patients with psychotic episodes due to a general medical condition, substance-induced psychotic disorder, intellectual disability, or psychotic episodes that were associated with major depressive disorder or bipolar disorder were excluded. In fact, 60 patients met the criteria for antipsychotic-naïve FEP with confirmed SCZ or schizophreniform disorder diagnoses after the follow-up and were included in this study analysis (N=60). These patients were assessed at baseline and were followed up for 9.03 ± 2.76 weeks of risperidone treatment.

The healthy control group (N=60) comprised age-and-gender-matched volunteers with no current or previous psychiatric diagnoses or first-degree family history of psychotic disorders.

Peripheral blood samples were collected in EDTA tubes at the baseline and the follow-up for patients and after psychiatric interview for controls.

Clinical assessments

FEP patients were assessed at both time points by: a) PANSS (Positive and negative syndrome scale) (Vessoni, 1993), b) CGI (Clinical global impression scale) (Lima et al., 2007), c) GAF (Global assessment of functioning scale), d) CDSS (Calgary Depression Scale for Schizophrenia) (Bressan et al., 1998).

Symptom clusters (Negative, Positive, Disorganization, Excited and Anxiety/Depression) were classified according to the PANSS ratings (Wallwork et al., 2012) and were derived from a previous study in a Brazilian population (Higuchi et al., 2014). For more information about each symptom cluster, see Supplementary Table S2. Response to treatment was defined as a reduction of more than 50% of baseline PANSS total score (Leucht et al., 2009). GAF is the only scale that higher values represent less impairment, thus, we transformed to them to negative values (referred as -GAF).

DNA isolation:

Whole blood was collected into EDTA tubes and genomic DNA isolation was performed using the Gentra Puregene Kit (Qiagen) according to the manufacturer's protocol.

Genomic arrays:

The genotyping was performed at King's College London using the Infinium PsychArray-24 BeadChip (Illumina) with a GWAS core backbone and specific content from the Psychiatric Genomics Consortium: https://www.med.unc.edu/pgc/psychchip.

Quality control and Imputation:

For the Quality Control (QC) parameters we removed SNPs with a Minor Allele Frequency (MAF) < 1%, *Locus* missingness > 10% or Hardy-Weinberg Equilibrium significance < 0.00001. We also excluded individuals with missingness > 10% and an estimation of Identity by Descent greater than 0.12. Genotype imputation was performed in the https://imputation.sanger.ac.uk using as Reference Panel the Haplotype Reference Consortium (release 1) with 32,488 samples (39 M sites) and the Pre-phasing algorithm SHAPEIT2. Post-imputation QC used the same parameters from above.

Polygenic Risk Scores (PRS):

For more information about how the scores are calculated, please see the Supplementary Material of (Purcell et al. 2009). To generate the PRS we used the PRSice software (**Error! Hyperlink reference not valid.** default options. The SCZ sample from PGC2 (downloaded from https://www.med.unc.edu/pgc) was set as training sample and our imputed genotyping sample as target sample. We performed *P*-value-informed clumping with a cut-off of r2 = 0.1 within a 250-kb window and

calculated scores per individual for multiple p-threshold (ranging from 0.0001 to 0.5 with increments of 0.00005) including or excluding the MHC (major histocompatibility complex) region on chromosome 6, which has a complex linkage disequilibrium structure. Given that our sample is from a mildly admixed south eastern Brazilian population, principal component analysis was used and the first four components were used as covariables. Posteriorly, PRSice runs a regression to find the best p-threshold based on the explained variance (Nagelkerke's correlation) and in our case giving PRSs based on the most FEP case-control variance explained.

Statistical Analysis:

We used the R software for all statistical analysis. Taking forward the PRSs calculated for the case-control comparison, we used a generalized linear model to test PRS associations assuming a Poisson distribution (Poisson regression), which is more suitable for ordinal variables (psychiatric scales), using the clinical traits as the dependent variable and the best p-threshold score and the first four principal components as the independent variables and covariables. As clinical outcome variables, we considered for both time points GAF score, total CGI score, total PANSS scores and the five PANSS dimension clusters suggested by Wallwork et al., (2012) (Wallwork et al., 2012) and validated by Higuchi et al., (2014) (Higuchi et al., 2014) to the Brazilian population. GAF values were transformed to negative values (-GAF), so all clinical variables are equally interpreted, with high values meaning high symptomatology. We applied a Bonferroni correction for multiple comparisons (number of psychiatric scales tested for each time point, N=12) and considered as significant a corrected p-value < 0.05.

Further, we tested if the response to Risperidone or the subtypes of FEP included in our study (SCZ or schizophreniform) were associated with SCZ PRS. First, we tested, for only for the statistically significant clinical variables above, the change in symptoms from baseline to the follow-up and if the subtype of FEP was associated with the PRS using a Poisson regression. Second, we tested the association between total PANSS and PRS using a t-test, categorizing as responders those with 50% reduction in total PANSS.

Results:

We present in Table 1 the clinical and demographic characteristics of the participants in this study. Smoking rates were significantly different between cases and controls, with more patients smoking than controls. Regarding clinical variables, patients improved after two months of risperidone treatment for all scales and symptom clusters, except for PANSS negative.

Polygenic risk:

The number of independent SNPs analysed for each threshold and cohort is described in Supplementary Table S1. With or without the MHC region the results were similar, thus, we carried on with the MHC region to increase the number of analysed SNPs and, consequently, the power of our analysis. The polygenic risk score was significantly different in cases and controls (Figure 1) with a best p-threshold of 0.0112 (N_{SNPs}=21,622) and an explained variance of 0.19 (Naegelkerk's p-value = 0.0001).

PRS and clinical variables relation:

At the baseline (antipsychotic naïve FEP), we found a positive association of the best PRS score with the PANSS excitement factor (five-factor model) (corrected p-value = 0.004) and with –GAF (corrected p-value = 0.039). The PRS was positively correlated to depressive symptoms at baseline (CDSS total corrected p-value = 0.047) but was negatively associated with depressive symptoms after risperidone treatment (CDSS total: corrected p-value = 0.006; PANSS Depressive/anxiety: corrected p-value = 0.017). The results are summarized in Table 2.

With respect to response to risperidone, we observed a positive association for the ΔPANSS excitement ($PANSS\ excitement_{baseline}$ – $PANSS\ excitement_{follow\text{-}up}$) (B = 473; p-value = 0.003) and ΔCDSS (B = 717; p-value = 0.0006), however, there was no difference between risperidone responders (N=20) and non-responders (N=32) in PRS (t = 1.11; df = 41.2; p = 0.272). Depressive symptoms are associated with PRS in both FEP subtypes (schizophrenia or schizophreniform), but PANSS excitement and -GAF is associated in the schizophreniform subgroup.

Discussion:

We have demonstrated that PRS generated from the PGC2 SCZ sample is applicable to Brazilian patients and that it is associated with clinical measurements during different stages in SCZ, including symptoms pre-treatment. Specifically, we identified a positive association at the baseline of PRS with depressive symptoms (CDSS total), excitement symptoms (PANSS-excitement factor) and with the Global Assessment of Functioning (-GAF). After treatment no positive association is observed for these or other clinical measurements, instead a negative association with PRS is seen for both CDSS and PANSS depressive/anxiety. That this seen with both scales that measure depressive symptoms, reinforces the consistency of our clinical data.

It is known that the prevalence of depression in SCZ may be as high as 61% (Gozdzik-Zelazny et al., 2011); and in FEP this rate may be as high as 80% (Upthegrove et al., 2010; Sonmez et al., 2016). Furthermore, a moderate to high genetic correlation between SCZ and major depressive disorder (MDD) is known from twin studies and has been also been reported from GWAS of SCZ and MDD (Lee et al., 2013), with SCZ PRS showing more predictive ability in early than adult onset MDD (Power et al., 2016). We also found an association between excitatory dimension and PRS for SCZ.

One strength of our study is that all patients were antipsychotic-naïve at the baseline and received the same treatment for relatively the same time, which allow us to observe the clinical features without a couple of confounding factors and within a more homogenous group. _ENREF_35The treatment used, risperidone, has been shown to be beneficial as an augmentation therapy in MDD patients who have a high-risk for suicide (Reeves et al., 2008) and in patients who were treatment-refractory for MDD (Mahmoud et al., 2007). In our sample, the FEP patients that present with lower depressive symptoms, or that respond well to risperidone, are those with a higher genetic risk for SCZ. Future studies could test if MDD patients with higher risk for suicide and/or treatment-refractory fit this model and have higher SCZ PRS.

It is important to note that all previous studies of PRS and symptoms in schizophrenia used different study designs and, moreover, their samples were composed by patients under antipsychotic treatment. One study has suggested an association between depressive symptoms and SCZ PRS for in an overall analysis

of a sample of treated SCZ cases and controls, although this may have been due to the increased rate of depression in SCZ cases as, when they analysed cases and controls separately, they did not find SCZ PRS significantly correlated with the depression dimension (Derks et al., 2012). A second study, within a large population cohort of adolescents, found an association between PRS and negative symptoms but not with depressive or excitement dimensions (Jones et al., 2016).

Recently, a study suggested that the different subgroups of FEP have different PRS (Vassos et al., 2016). Although we did not observe overall PRS differences between our FEP subgroups (which were somewhat different to the Vassos et al study), we found our positive association with PANSS excitement and (-)GAF was driven by the subgroup composed by individuals with schizophreniform or brief psychosis disorder diagnoses. This might suggest an effect of shorter duration of symptoms, as both of these categories do not reach criteria for psychosis considering the 6 months requirement in DSMIV criteria. Our data also suggest the observed associations between dimensions of symptoms and PRS might change between different stages of the disorder._ENREF_30_ENREF_22

In terms of prediction of treatment response, we found that baseline to follow-up changes for PANSS excitement and CDSS were also positively associated with PRS for SCZ, suggesting that patients with a higher PRS tend to show more improvement in symptoms after treatment. Considering the post treatment timepoint alone, the association between PRS and clinical variables is not observed. Also, we detected differences in PANSS dimensions, but not when we divided the sample into responders and non-responders, but our power decreases markedly when grouping patients into two categories.

Our study has several limitations, primary amongst which is that our FEP sample size is small (N=60). However, it is a unique longitudinal sample of antipsychotic naïve First Episode of Psychosis individuals. We will increase this sample in the future but at the moment it represents a (near) unique resource. Despite these limitations, this is the first study to explore the PRS relation to multiple clinical traits measured before standardized treatment initiation in the FEP as well as the change in symptoms after a sufficient time period in which to observe response to treatment.

We expect that future studies explore the clinical dimensions taking into account the response to the different antipsychotics, increasing the sample size

analyzed will have more power to detect potential correlations between clinical data and PRS during FEP phase. Despite these limitations, this is the first study to explore the PRS relation to multiple clinical traits during the FEP and a few weeks after the implement of antipsychotic drugs. In conclusion, we have shown that that excitement and depressive symptoms are positively associated to PRS SCZ during FEP baseline but not after risperidone treatment and that the PRS SCZ predicts response to treatment.

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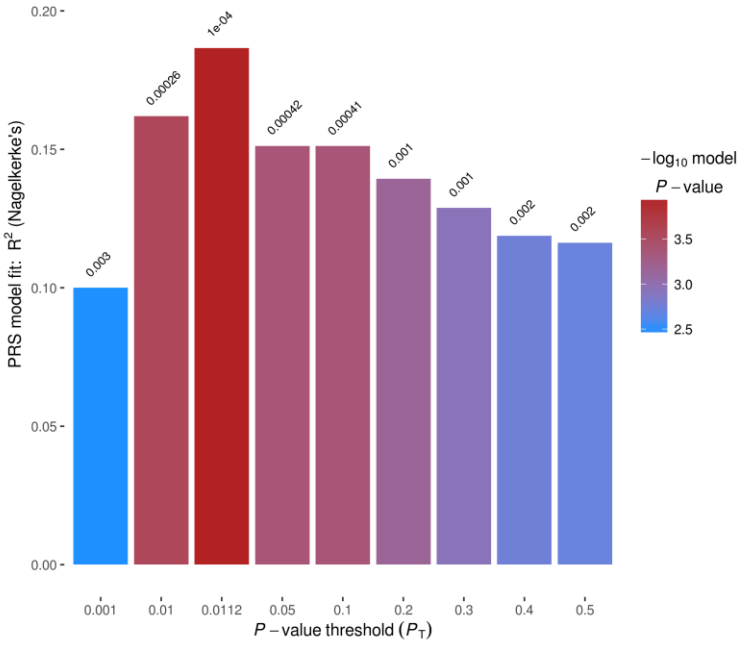

Figure 5. Graph from PRSice showing the explained variance (y-axis) for each p-threshold (x-axis) to identify cases and controls for our sample.

Table 1: Clinical and demographic characteristics of the participants in this study.

Variable	Healthy controls (N=59)	Antipsychotic-naïve FEP (N=60)	FEP after treatment (N=60)	p-value
Gender (%)	M:34 (57.6%) F:25 (42.4%)	M:40 (66.7%) F:20 (33.3%)		0.309
Age in years; mean (SD)	25.97 (7.48)	25.63 (7.46)		0.808
Currently smoking (%)	Yes: 2 (3.6%) No: 54 (96.4%)	Yes: 12 (23.5%) No: 39 (76.5%)		0.002
PANSS negative; mean (SD)		27.37 (10.51)	25.02 (9.28)	0.127
PANSS disorganization/cognition; mean (SD)		26.96 (8.58)	19.91 (6.22)	5.029×10^{-8}
PANSS excitement; mean (SD)		24.69 (9.09)	13.22 (5.62)	7.84×10^{-14}
PANSS positive; mean (SD)		34.75 (7.32)	21.23 (9.52)	2.46×10^{-13}
PANSS depression/anxiety; mean (SD)		24.24 (8.79)	18.11 (7.85)	2.97×10^{-5}
PANSS total		94.55 (20.94)	68.21 (20.31)	1.71×10^{-10}
GAF; mean (SD)		31.21 (10.52)	55.47 (16.61)	1.34×10^{-11}
CGI; mean (SD)		4.83 (0.72)	3.35 (1.26)	1.77×10^{-11}
CDSS; mean (SD)		4.64 (5.04)	2.48 (4.27)	0.007

M: male; F: female; SD: Standard deviation; FEP: First-episode psychosis; PANSS: Positive and Negative Syndrome Scale; CGI: Clinical Global Impression Scale; GAF: Global Assessment of Functioning Scale; CDSS: Calgary Depression Scale for Schizophrenia

Supplementary Table S1. PRSice results, explained variability and number of SNPs for each threshold.

Threshold	p.out (Naegelkerke's)	r2.out	Nsnps
0.001	0.0032	0.10	5533
0.01	0.0003	0.16	20223
0.0112	0.0001	0.19	21622
0.05	0.0004	0.15	53790
0.1	0.0004	0.15	82978
0.2	0.0007	0.14	126514
0.3	0.0011	0.13	160713
0.4	0.0018	0.12	188576
0.5	0.002	0.12	211371

Table 2. PRS correlation with clinical variable during the baseline and the follow-up

Time	Clinical variable		N	B	Raw p-value	Bonferroni
Baseline (antipsychotic naive FEP)	CGI		50	72.8	0.8436	1.000
	* -GAF		48	436.1	0.0030	0.039
	* CDSS Total		51	1042.3	0.0039	0.047
	PANSS Total		53	38.1	0.6390	1.000
	PANSS Positive		53	400.0	0.0278	0.361
	PANSS Negative		53	-205.6	0.3048	1.000
	PANSS General Psychopathology		53	-20.2	0.8886	1.000
	Five-Factor Model (Higuchi et al., 2014)	PANSS negative	53	-168.2	0.2655	1.000
		PANSS Disorganization / cognition	53	-32.9	0.8281	1.000
		* PANSS excitement	53	566.7	0.0003	0.004
		PANSS positive	53	27.1	0.8382	1.000
		PANSS depression / anxiety	53	-112.8	0.4761	1.000
Follow-up (two months treated with risperidone)	CGI		51	-137.4	0.7588	1.000
	-GAF		53	-132.8	0.2281	1.000
	* CDSS Total		53	-1800.2	0.0004	0.006
	PANSS Total		54	-113.4	0.2215	1.000
	PANSS Positive		56	277.3	0.3141	1.000
	PANSS Negative		56	-358.3	0.0894	1.000
	PANSS General Psychopathology		54	-287.9	0.1140	1.000
	Five-Factor Model (Higuchi et al., 2014)	PANSS negative	56	-180.6	0.2329	1.000
		PANSS Disorganization / cognition	56	-75.8	0.6608	1.000
		PANSS excitement	56	216.4	0.3048	1.000
		PANSS positive	56	10.0	0.9522	1.000
		* PANSS depression / anxiety	55	-575.0	0.0013	0.017

PANSS: Positive and Negative Syndrome Scale; CGI: Clinical Global Impression Scale; GAF: Global Assessment of Functioning Scale; CDSS: Calgary Depression Scale for Schizophrenia

Table S2. Five-factor model.

Five-Factor Model	ITEMS	Question
NEGATIVE	Poor rapport	N3
	Lack of spontaneity	N6
	Emotional withdrawal	N2
	Passive/apathetic social withdrawal	N4
	Blunted affect	N1
	Motor retardation	G7
DISORGANIZATION / COGNITION	Conceptual disorganization	P2
	Poor attention	G11
	Disorientation	G10
	Disturbance of volition	G13
	Difficulty in abstract thinking	N5
	Stereotyped thinking	N7
	Mannerisms/posturing	G5
EXCITEMENT	Uncooperativeness	G8
	Poor impulse control	G14
	Hostility	P7
	Excitement	P4
POSITIVE	Delusions	P1
	Unusual thought content	G9
	Hallucinatory behavior	P3
	Suspiciousness/persecution	P6
	Grandiosity	P5
DEPRESSION / ANXIETY	Anxiety	G2
	Guilt feelings	G3
	Depression	G6
	Tension	G4

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5.5 **Artigo 4 – Santoro et al., (em preparo)**

Objetivos: Identificar marcadores genéticos utilizando análise transcriptômica e metilômica em uma coorte de PEP antes e após o uso de risperidona.

Casuística e metodologia: Para este estudo foram selecionados 60 controles e 60 pacientes em PEP antes (pacientes virgens de tratamento com antipsicótico) e após dois meses de risperidona. Todos os pacientes têm entre 18-40 anos e preencheram os critérios de diagnósticos psicóticos de acordo com o DSM-IV. O sangue foi coletado para a extração de DNA e de RNA. Geramos os dados de expressão gênica utilizando o *microarray* de expressão *Illumina HT-12 BeadChip*. Para os dados de metilação de DNA, utilizamos o *microarray Illumina methylathion Human 450K BeadChip*. Para todas as análises utilizamos como covariável os seguintes dados: 4 primeiros componentes principais da genotipagem dos indivíduos, sexo, idade e tabagismo.

Resultados: Após as correções, nós não identificamos nenhum gene diferencialmente expresso entre PEP e controles, contudo encontramos 15 genes diferencialmente expressos entre o PEP antes e após o tratamento ($p<8.4E^{-06}$). Com relação a regiões diferencialmente metiladas (DMRs), nós encontramos cinco DMRs entre PEP e controles e 10 entre PEP antes e após o tratamento ($p <7.76E^{-06}$).

Discussão: Neste estudo identificamos pela primeira vez regiões diferencialmente metiladas em uma coorte longitudinal de pacientes em PEP virgens tratamento com antipsicóticos. Tanto alguns genes diferencialmente expressos quanto algumas regiões diferencialmente metiladas encontradas nesses estudo, já foram associados com a esquizofrenia no maior GWAS para a doença. Além disso, coletivamente, os resultados apontam que as alterações entre PEP antes e após o tratamento estão relacionadas com os sintomas adversos da risperidona.

Genome-Wide Expression and DNA Methylation Analysis in a Longitudinal Cohort of Antipsychotic-Naive First Episode of Psychosis patients

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Abstract

In this study we aimed to identify genetic markers, using whole genome expression and DNA methylation approaches in a FEP cohort before and after the use of risperidone.

Sixty controls and sixty patients FEP before (antipsychotic naïve) and after two months of risperidone (FEP-2M) were recruited for this study. All patients were 18-40 years and have met the criteria of psychotic diagnoses according to DSM-IV. Blood was collected for DNA and RNA isolation. We generated gene expression data using the Illumina HT-12 BeadChip microarray. For the DNA methylation data, we used the Human Illumina 450K BeadChip microarray. For all analyses, we used as covariate: the four principal components of genotyping, sex, age, smoking and cell type (deconvolution). We considered as statistically significant those differentially expressed genes (DEGs) and differentially methylated regions (DMRs) with p value lower than 0.05 after a Bonferroni correction for multiple comparisons (number of genes or methylated regions). We did not identify any DEG between FEP and controls, but we found 15 DEGs between the FEP before and after treatment ($p < 8.4E^{-06}$). Concerning DNA methylation, we found five DMRs between controls and FEP, and ten DMRs between FEP before and after treatment ($p < 7.76E^{-06}$). To our knowledge, this is the first study to find DMRs in a longitudinal cohort of antipsychotic naïve FEP patients. Collectively, the DEGs and DMRs in the longitudinal comparison seem to be related to the adverse effects and the response to risperidone. Curiously, the most associated genomic region in schizophrenia (previously reported by the PGC), the MHC region, is less methylated in patients after the treatment, demonstrating an epigenetic modification caused by risperidone in an important schizophrenia genomic region. Identifying the genetic and molecular changes of drug response is one of the first steps through personalized

medicine, further studies should replicate these results and aim antipsychotic naive individuals treated with other APDs.

INTRODUCTION

Schizophrenia is a severe and multifactorial psychiatric disorder that is characterized by diverse psychopathology, including the psychotic symptoms (e.g. delusions and hallucinations), negative symptoms (e.g. impaired motivation, reduction in spontaneous speech, and social withdrawal) and cognitive impairment (Owen et al., 2016). Antipsychotic drugs (APDs) are effective in treating positive symptoms, improving outcomes and reducing relapses rates (Taylor et al., 2012), but may cause adverse effects. Typical APDs, such as haloperidol, act mainly on dopaminergic receptors, and, depending on the potency and dose used, can induce extrapyramidal symptoms. Conversely, atypical APDs, for example risperidone, target a wide range of neurotransmitter receptors including dopaminergic, serotonergic and muscarinic receptors and tend to cause metabolic side effects (Miyamoto et al., 2005; Lieberman et al., 2008).

The study of the early stages of schizophrenia, the first-episode psychosis (FEP), is critical given that brain abnormalities and cognitive deficits are already present (Demjaha et al., 2012). Moreover, because FEP individuals have a shorter period of medication and duration of symptoms, they might be considered as a more naive cohort compared to chronic patients (Fuste et al., 2013).

Heritability estimate for schizophrenia is roughly 80%, indicating that genetic factors play an important role in the underlying cause of the disease but are not sufficient (Owen et al., 2016). Many genetic studies have been conducted, confirming that schizophrenia is highly polygenic. The largest genome-wide association study (GWAS) to date revealed 108 loci associated with this disorder (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014); however, very few variants were non-synonymous (change in protein sequence), suggesting that most of the GWAS findings may alter gene expression rather than protein structure. Therefore, additional approaches, including gene expression and DNA methylation studies, that also reflect environmental alterations (Turecki et al., 2014) are necessary to further understand the molecular underpinnings of schizophrenia

A previous genome-wide RNA sequencing study observed differential mRNA levels of 200 genes in blood of 36 antipsychotic-naïve FEP patients compared with 40 controls (Sainz et al., 2013). Moreover, by comparing 22 patients before and after

treatment with antipsychotic medications, the expression level of 17 genes were altered (Crespo-Facorro et al., 2014). Most of these genes are involved in inflammatory and immune response, and some were previously implicated in schizophrenia or bipolar disorder (Sainz et al., 2013; Crespo-Facorro et al., 2014). Therefore, this and other studies indicate a potential confounding effect of antipsychotic treatment in gene expression studies in individuals with schizophrenia (Ota et al., 2014a; Ota et al., 2015).

APDs and psychotic disorders also seem to influence DNA methylation, which is an important epigenetic modification involved in the regulation of transcription, in different tissues (Guidotti and Grayson, 2014; Ota et al., 2014b; Santoro et al., 2014). In a large sample of 1497 schizophrenia cases and controls, Aberg et al. (2014) found 139 differentially methylated sites in blood related to hypoxia and immune system (Aberg et al., 2014). However, analyses in chronic schizophrenia patients may not be applicable to patients with FEP, and very few studies were conducted in these patients. In blood cells, Nishioka et al (2013) identified a global hypomethylation in 18 patients with first-episode schizophrenia compared to 15 normal controls. Moreover, genes related to the nuclear lumen, to nucleotide binding and to transcription factor binding were also hypomethylated in patients (Nishioka et al., 2013). Investigating prospectively DNA methylation during psychosis transition in ultra-high risk individuals, Kebir et al. (2016) reported alterations of methylation during conversion to psychosis in gene promoters and pathways relevant for psychosis, including oxidative stress regulation, axon guidance and inflammatory pathways (Kebir et al., 2016).

In this study we aimed to identify genetic markers using whole genome expression and DNA methylation approaches in a longitudinal FEP cohort before and after the use of risperidone.

Methods

Study population

Antipsychotic-naïve FEP patients (N=60) were recruited from a psychiatric emergency unit in São Paulo, Brazil. The diagnosis of a psychotic disorder was established by trained psychiatrists based on the criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) using the Structured Clinical Interview of the DSM-IV (SCID-I). All patients fulfilled the criteria for one of the

following psychotic diagnoses according to the DSM-IV: schizophrenia, schizophreniform disorder, brief psychotic disorder and psychotic disorder not otherwise specified.

Inclusion criteria included age between 16 and 40 years and no prior history of antipsychotic medication. Patients with psychotic episodes due to a general medical condition, substance-induced psychotic disorder, intellectual disability or psychotic episodes that were associated with bipolar or major depressive disorders were excluded.

The patients were evaluated at baseline and after two months of follow-up using a) PANSS (Positive and Negative Syndrome Scale), b) CGI (Clinical Global Impression Scale), c) GAF (Global Assessment of Functioning Scale), and d) CDSS (Calgary Depression Scale for Schizophrenia). PANSS dimensions were derived from a previous study with Brazilian population (Higuchi et al., 2014). Treatment response was defined as a reduction of 50% in PANSS total scores after discounting the 30 minimum points of the total PANSS score (Leucht et al., 2009).

Peripheral whole blood samples were collected at baseline (N= 60) and after 9.82 weeks (SD=3.34) of risperidone treatment (FEP-2M) (N=60). Risperidone was standardized at doses between 1 and 6 mg based on clinical need. The healthy control subjects (N=60) were age- and gender-matched volunteers who had no current or previous psychiatric diagnoses or first-degree family history of severe psychiatric disorder.

The Research Ethics Committee of UNIFESP approved the research protocol, and all participants and family members provided written informed consent prior to enrolment in the study (CEP 0603/10 and CAAE 33148114.6.0000.5505).

DNA and RNA isolation

Whole blood was collected into EDTA tubes and genomic DNA isolation was performed using the Gentra Puregene Kit (Qiagen, Maryland, USA) according to the manufacturer's protocol.

Gene Expression analysis

The RNA samples were defrosted, quantified and the quality assessed with Bioanalyzer (Agilent, Germany) to ensure that samples were not degraded. The average RNA Integrity Number (RIN) was 9.1 and the lowest was 6.6. To verify the

whole gene expression, we used the HumanHT-12 v4 Expression BeadChip (Illumina), which interrogates approx. 25,000 genes containing more than 48,000 probes. The content of this BeadChip provides genome-wide transcriptional coverage of well-characterized genes, gene candidates, and splice variants, delivering high-throughput processing of 12 samples per BeadChip. HumanHT-12 Expression BeadChips use the Direct Hybridization Assay and was scanned on the iScan systems (Illumina) of the SGDP lab. We followed the manufacturer's protocol.

Initial quality control

The results are downloaded from the iScan and preanalyzed using the GenomeStudio software. First, we checked the average signal and the number of detected genes for each sample, excluding those deviate substantially from the main chord of data points.

PreProcessing probes and samples

After the quality control, we performed a background correction using the Maximum Likelihood Estimation (MLE) of the Model-Based Background Correction R package (Allen et al., 2009). To ensure that the different BeadChips are comparable among each other, we used a robust spline normalization (RSN), which combines the features of quantile and loess normalization and is designed to normalize the variance-stabilized data. Finally, we identified the potential batch effects and corrected for the RIN, the input cRNA concentration and the barcode of each chip.

Principal Components from genotyping data

The data from ten principal components was generated to reflect different ancestry dimensions in our sample, the we used the first four principal components. For details, see Santoro et al., submitted to JAMA Psychiatry.

Differentially Expressed Genes (DEGs)

To identify genes differentially expressed (DEGs) we used the Linear Models for Microarray Data (Limma) R Package. It estimates the fold changes and standard errors by fitting a linear model for each gene and then, applies empirical Bayes smoothing to the standard errors. We used the basic two groups analysis to find DEGs between FEP x HC and the Paired Sample option in the comparison between FEP x FEP-2M. For more information about the method, please see:

https://www.bioconductor.org/packages/3.3/bioc/vignettes/limma/inst/doc/usersguide.pdf

We used the R package "CellMix" to predict the cell type proportion for each individual. For both analyses, we used as covariates age, sex, smoking data, cell type proportions and the four principal components mentioned above. Finally, we used a Bonferroni correction for multiple comparisons, considering as significant those genes with a p-value lower than 0.05.

Methylation Array

Concerning DNA methylation analysis, we generated data using the Infinium HumanMethylation450 BeadChip Kit. This BeadChip has a 12-sample per array format that interrogates > 485,000 methylation sites per sample at single-nucleotide resolution, covering 99% of RefSeq genes. We followed the manufacturer's protocol and their prerequisites to prepare the samples.

Preprocessing

The raw files generated were then processed using the wateRmelon R package. First, samples with 1% of sites with a detection p-value greater than 0.05 are excluded and then, the probes are filtered if beadcount is lower than 3 in 5 % of samples. After that, we used the "*dasen*" method to process the probes, which performs a background adjustment followed by a between-array normalization applied to Type I and Type II probes separately.

Differentially Methylated Positions (DMP) and Differentially Methylated Regions (DMRs)

We used the same covariates that we did for DEG analysis. To find DMPs (differentially methylated CpG sites), we used the beta values after the preprocessing and applied a similar analysis to that described for DEG. We used limma R package, using the same two separated analyses.

To calculate the DMRs we used the DMRcate R software. It uses the unadjusted p-values from the DMPs (all sites with a p-value lower than 0.05), (1) calculates auto-correlation, (2) combines adjacent P-values, (3) performs false discovery adjustment, (4) finds regions of enrichment (i.e. series of adjacent low P-values) and (5) assigns significance to those regions. We considered as a significant DMR if there were at least

5 significant sites within this region and with a maximum distance between them of 500 bp. All DMRs with an adjusted p-value are reported as significant in the results.

Ricopilli database

Ricopilli is an online database that compiles all PGC recently published GWAS results. For gene expression and DNA methylation results, we checked if the genes and/or regions found by our study was previously identified in SCZ PGC2 GWAS. We considered as an indicative hit if a region has more than 5 independent SNPs associated to the disease with a p-value lower than $1x10^{-5}$ (and not the classical GWAS threshold of $p<1x10^{-8}$).

Results

DEGs

After the preprocessing, 166 samples (57 HC, 55 FEP and 54 FEP-2M) and 5935 probes remained for further analysis. We found fifteen genes differentially expressed between the FEP before and after the risperidone treatment, and no genes differentially expressed between FEPs and controls (supplementary material). Table 1 shows the DEGs.

Table 2. DEGs between FEP patients before and after the risperidone treatment.

Gene	Groups	Ricopilli hit ($p<1x10^{-5}$)	Raw p-value	Bonferroni p-value	Fold Change	Function
C1orf128	FEP x FEP-2M	No	$4.08E^{-07}$	0.002	0.53	megakaryocyte differentiation
ADIPOR1	FEP x FEP-2M	No	$1.37E^{-06}$	0.008	0.52	receptor of adiponectin
GMPR	FEP x FEP-2M	No	$2.10E^{-06}$	0.012	0.69	deamination of GMP to IMP
SNCA	FEP x FEP-2M	No	$2.27E^{-06}$	0.013	0.73	major component of Lewy bodies
WDR40A	FEP x FEP-2M	Yes	$3.42E^{-06}$	0.020	0.55	----
TESC	FEP x FEP-2M	No	$4.98E^{-06}$	0.029	0.58	calcium ion binding pathway
MAP2K3	FEP x FEP-2M	No	$5.31E^{-06}$	0.031	0.38	glucose transporter cofactor
ST6GALN	FEP x	No	$5.43E^{-06}$	0.032	0.57	glycosyltransfera

AC4	FEP-2M					se
DCAF6	FEP x FEP-2M	No	$5.51E^{-06}$	0.032	0.35	----
HAGH	FEP x FEP-2M	No	$6.21E^{-06}$	0.037	0.54	Carbonyl stress protection
BCL2L1	FEP x FEP-2M	No	$6.25E^{-06}$	0.037	0.5	anti/pro-apoptotic regulator
FBXO7	FEP x FEP-2M	No	$6.35E^{-06}$	0.037	0.56	----
FAM104A	FEP x FEP-2M	No	$6.35E^{-06}$	0.038	0.37	----
DMTN	FEP x FEP-2M	No	$7.65E^{-06}$	0.045	0.67	----
MPP1	FEP x FEP-2M	No	$8.14E^{-06}$	0.048	0.4	regulator of neutrophil polarity

DNA methylation:

In FEP x Control comparison no single sites (DMPs) were significant after all corrections, but 5 DMRs were significantly deregulated in FEP group, the most significant one is close to *RASA3* gene which is in an enriched association region for schizophrenia (Ricopili p<1x10-6).

For FEPxFollow-up, five DMPs and ten DMRs were statistically significant. Curiously, one DMP and one DMR is inside the MHC region, which is the most associated region in SCZ (ricopili $p<10x^{-18}$). Table 2 shows the most statistically significant DMRs.

Table 3. DMRs between FEP and control and between FEP before and after risperidone treatment.

Methylation Coordinate	Closest Gene	Groups	N.cpgs	minfdr	Mean betafc	Ricopili
chr1:92099528-92100082	--	FEP x FEP-2M	3	$8.6E-^{07}$	-0.018	No
chr19:58570419-58570995	ZNF606	FEP x FEP-2M	8	$1.3E^{-06}$	-0.005	No
chr8:47015640-47015868	--	FEP x FEP-2M	4	$2.8E^{-07}$	-0.012	No
chr1:92414221-92415145	BRDT	FEP x FEP-2M	10	$3.4E^{-08}$	0.0084	No

chr17:56769387-56769767	TEX14 / RAD51C	FEP x FEP-2M	8	$3.2E^{-06}$	-0.001	Yes
chr19:21933386-21933659	ZNF100	FEP x FEP-2M	6	$2.9E^{-06}$	-0.008	No
chr18:51884982-51885093	C18orf54	FEP x FEP-2M	4	$2.1E^{-05}$	0.0101	No
chr17:58499700-58499911	C17orf64	FEP x FEP-2M	6	$2.2E^{-05}$	0.0031	No
chr2:54086854-54087552	ASB3 / GPR75	FEP x FEP-2M	13	$3.4E^{-08}$	-0.009	Yes
chr6:33130918-33131560	COL11A2	FEP x FEP-2M	9	$1.7E^{-06}$	0.0011	Yes
chr13:114875001-114875412	RASA3	FEP x Controls	5	$4.7E^{-05}$	0.0284	Yes
chr11:1463541-1463935	BRSK2	FEP x Controls	5	$2.8E^{-04}$	0.0566	No
chr6:53530503-53530628	KLHL31	FEP x Controls	6	$3.1E^{-04}$	0.0161	No
chr11:5617273-5617812	TRIM6	FEP x Controls	6	$3.1E^{-04}$	0.0407	No
chr12:75784617-75785295	GLIPR1L2 / CAPS2	FEP x Controls	10	$2.8E^{-04}$	-0.021	No

DISCUSSION

To our knowledge, this the first study to evaluate the whole-genome transcriptional and DNA methylation profile concomitantly in a longitudinal cohort of FEP antipsychotic naïve at the baseline.

In the comparison between FEP and the controls we did not find any DEG, however, some of the top DEGs have been reported elsewhere (Supplementary Material Table 1) altered between SCZ chronic patients (De Jong et al., 2012) or FEP patients (Sainz et al., 2013). It is worth to note in this study we were very cautious and very strict in using many covariates (age, sex, smoking data, PC from genotyping, cell-type deconvolution) and Bonferroni correction, while previously studies have used less covariates and FDR correction.

Concerning the methylation results, we identified five DMRs between FEP and controls. One of this region presented a mild association with SCZ in the last PGC GWAS ($p<6x10^{-6}$), however, there is a lack of studies that explored the function of this gene and the other four genes close to the DMRs. Curiously, *BRSK2* gene methylation

has been reported as marker of epigenetic variability across human populations. Although we have used the same covariates described above, including the PC of genotyping, which should soften the differences among populations in our sample, we cannot exclude that population stratification is influencing our results between FEP and controls.

Concerning the longitudinal comparison, we identified 15 DEGs and 10 DMRs that have their pattern changed after two months of risperidone treatment. Curiously, three genes and one region (*GMPR; ADIPOR1; MAP2K3*) has been implicated with obesity related traits (Chasman et al., 2009; Comuzzie et al., 2012; Bian et al., 2013; Lee and Hung, 2015), and all of them are down regulated in the group after treatment. Curiously, Adiponectin receptor (*ADIPOR1*) down-regulation has been implicated in obesity (Yamauchi et al., 2007). Since one of the risperidone adverse effects is weight gain (Almandil et al., 2013), we suggest these alterations found in genes related to obesity might be related to risperidone adverse effect. Although it is a quite interesting result, we do not have the weight or BMI data of these patients at both timepoints to test the correlation with gene expression.

Similarly, we identified two genes previously implicated in Parkinson disease (*SNCA* and *FBXO7*). While SNCA is deemed as a major component of Lewy bodies, a neuropathological feature of Parkinson disease (Vilar et al., 2008), some *FBXO7* mutations have been implicated in young-onset parkinsonism (Di Fonzo et al., 2009). Indeed, both genes seem to be involved in the same process of neurodegeneration (Zhao et al., 2012). Here, we found that both genes are down regulated after the treatment, suggesting that risperidone might be acting in this neurodegenerative pathway. Even though risperidone is a second generation APD, and thus, present less adverse effects, a review reported that risperidone produce more extrapyramidal side effects (including parkinsonism) than other atypical APD (Komossa et al., 2011).

BCL2L1 has two splice variants that can act as anti or pro-apoptotic regulators (Boise et al., 1993). In this study, we found a decrease in *BCL2L1* gene expression in FEP-2M. Similarly, a decrease of *BCL2L1* gene expression (Fatemi et al., 2012) and protein levels (He et al., 2006) were reported in rats brain treated with antipsychotics and suggested to be related to the antipsychotic efficacy.

Still concerning the longitudinal comparison, we found that one of the DMRs is inside the MHC region which has been consistently associated to SCZ (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014) when compared to controls, however, this association has not yet been fully explained. The DMR we identified is less methylated in the FEP-2M compared to antipsychotic naïve FEP and is exactly at the end of this MHC region, close the *COL11A2* gene and *HLA-DPB2*.

This study has some limitations that should be addressed. First, our FEP sample size is small (N=60), however, it is a unique longitudinal sample of antipsychotic naïve First Episode of Psychosis individuals. Second, all the results refer to DMRs and DEGs found in blood of patients and we cannot extrapolate that same DMRs and DEGs are altered in brain regions, but they can still be considered peripheral markers, though. Third, we did not control for metabolic changes, a known risperidone side effect, which could have affected our results.

In conclusion, our study demonstrates the advantages in working with a longitudinal cohort with a standardized treatment protocol, which allowed us, even with a small sample size, identify altered DMRs and DEGs between FEP individuals before and after the treatment. In general, our results pointed to DMRs and DEGs that might be related to risperidone side effects or response to treatment. Identifying the genetic and molecular changes of drug response is one of the first steps through personalized medicine, further studies should replicate these results and aim antipsychotic naive individuals treated with other APDs.

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6 LIMITAÇÕES DO ESTUDO

Abaixo, enumeramos as principais limitações do presente estudo:

Limitações comuns aos quatro artigos

O número de amostras avaliadas, especialmente de indivíduos EMR, foi pequeno e, com isso, algumas análises estatísticas apresentaram baixo poder. Dessa forma, os nossos achados dever ser tratados com cautela e preferencialmente replicados em outras coortes. O N amostral baixo é consequência da dificuldade em identificar sinais precoces de psicose em adolescentes e adultos jovens e encaminhá-los para serviços de atendimento. Além disso, atualmente o PRISMA é o único centro de pesquisa no Brasil especializado no reconhecimento de indivíduos em EMR para psicose, o que não possibilita a parceria com outros grupos nacionais com profissionais devidamente treinados para identificar esses indivíduos.

Com relação ao grupo de pacientes em PEP, utilizamos como principal critério de seleção o fato dos indivíduos serem virgens de tratamento com antipsicótico. Embora esse fato aumente a robustez da nossa coorte, ele também diminui o tamanho amostral.

Todo o material biológico utilizado nesse estudo (RNA e DNA) é proveniente de sangue periférico. Apesar da possibilidade em se detectar marcadores em sangue, seria muito importante compreender como as alterações no sangue estão influenciando o tecido cerebral durante as diferentes fases da esquizofrenia, o que hoje não é possível de ser realizado em humanos.

O recrutamento de controles para os dois estudos foi realizado em grande parte na Universidade Federal de São Paulo, sendo que boa parte dos indivíduos são alunos e funcionários deste local. Desta forma, a amostra de controles apresenta um padrão sócio demográfico diferente dos pacientes PEP e e dos indivíduos EMR. Apesar de termos pareado por idade e sexo, essa diferença populacional das duas amostras pode ter influenciado nossos resultados.

Limitações exclusiva do artigo 1

Indivíduos EMR foram seguidos por um curto período de tempo (média de seguimento de 1 ano) e apenas dois pacientes converteram para esquizofrenia (não incluídos no grupo FEP). Levando em consideração que a maior taxa de conversão para psicose já observada é de 30%, boa parte dos indivíduos estudados não irão converter para psicose e podem ter influenciado nossos resultados. Estudos longitudinais em outras coortes semelhantes precisam ser feitos para replicar esses resultados.

Outra limitação exclusiva do artigo 1 é que cinco dos 22 pacientes em EMR estavam utilizando antipsicótico no dia da coleta de sangue, três com risperidona e outros dois com quetiapina. Dessa forma, também podemos sugerir que o aumento na expressão de *UFD1L* e de *MBP* pode ser derivado da administração precoce de antipsicótico, contrapondo com os indivíduos controles e pacientes em PEP, que são virgens para o uso de antipsicóticos. Apesar do número amostral reduzido, nós não encontramos diferenças estatísticas na expressão gênica entre esses cinco indivíduos em EMR sob efeito de antipsicótico e os demais 17 indivíduos desse grupo.

Limitação exclusiva do artigo 2

A revisão da literatura realizada nesse artigo foi conceitual e não uma revisão sistemática. Demos prioridade para os estudos GWAS com maiores tamanhos amostrais publicados nos últimos 5 anos.

Limitações exclusivas do artigo 3 e 4

Recentemente um outro estudo (Martin et al., 2014) mostrou que os indivíduos com PRS mais elevados para a esquizofrenia são mais suscetíveis a interromper estudos longitudinais e que esses indivíduos não respondem aos questionários adequadamente. Uma vez que um dos critérios de inclusão em nosso estudo era ter duas avaliações psiquiátricas, ou seja, ser acompanhado por pelo menos dois meses, a nossa coorte pode estar sub-representada por indivíduos com um alto PRS para esquizofrenia.

5. CONCLUSÕES

6.1 **Gerais**

- Identificamos em sangue periférico potenciais marcadores de estado da doença, ou seja, marcadores que parecem estar alterados em estágios específicos da esquizofrenia e de acordo com a exposição a antipsicóticos.
- Identificamos em sangue periférico potenciais marcadores da doença, ou seja, traços genéticos específicos entre controles e as fases iniciais da esquizofrenia.

6.2 **Estudo 1:**

- Encontramos genes diferencialmente expressos em sangue periférico entre EMR e PEP que possuem funções relacionadas com o neurodesenvolvimento e que têm sido associados à esquizofrenia.
- A expressão gênica de *UFD1L* está aumentada no grupo EMR quando comparado ao grupo controle e ao grupo PEP, sugerindo uma alteração específica para esse grupo.
- Tanto o aumento da expressão de *DISC1* (não significante após correção para idade) no grupo EMR como a diminuição da a expressão de *MBP* parecem estar associadas a um processo comum.

6.3 **Estudo 2: Conclusão geral**

Utilizando técnicas genômicas, transcriptômicas e epigenômicas em larga escala nós identificamos em sangue periférico genes e regiões associadas com o risco, progressão e resposta ao tratamento com risperidona.

6.3.1 Conclusões específicas

Relação de variáveis clínicas de gravidade e resposta ao tratamento com o PRS

- Nos pacientes em PEP virgens de tratamento nós observamos uma relação positiva entre o PRS e três medidas psiquiátricas (GAF, CDSS e PANSS-excitement). Para todas essas quanto maior o escore, maior era a gravidade dos

sintomas medidos. Após dois meses de tratamento com risperidona essa associação não era mais observada.

- Após o tratamento com Risperidona por dois meses nós observamos uma correlação negativa entre PRS e sintomas depressivos (avaliados pela PANSS e pela CDSS). Em outras palavras, aqueles pacientes com um PRS mais alto para esquizofrenia apresentam menos sintomas depressivos após o tratamento. O fato de encontrar resultados semelhantes para a CDSS e sintomas negativos da PANSS reforçam a coerência em nossas avaliações psiquiátricas.

Análise de transcriptoma e metiloma do PEP antes e após o tratamento com Risperidona

- Identificamos pela primeira vez regiões diferencialmente metiladas em uma coorte longitudinal de pacientes em PEP virgens de tratamento com antipsicóticos.
- Tanto alguns genes diferencialmente expressos e quanto algumas regiões diferencialmente metiladas encontradas nesses estudo já foram associados com a esquizofrenia no maior GWAS já realizado para a doença.
- Coletivamente, os resultados apontam para alterações relacionadas com sintomas adversos do tratamento com risperidona.

7 ANEXOS

7.1 Anexo 1: Aprovação do Comitê de Ética em Pesquisa da Unifesp: Doutorado

UNIVERSIDADE FEDERAL DE SÃO PAULO - UNIFESP/ HOSPITAL SÃO PAULO

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise de expressão gênica de mRNA e miRNA na busca de potenciais marcadores de risco e de conversão para a psicose em indivíduos em estado mental de risco (EMR)

Pesquisador: Marcos Leite Santoro

Área Temática: Área 1. Genética Humana.
(Trata-se de pesquisa envolvendo genética humana não contemplada acima.);

Versão: 2

CAAE: 06624613.1.1001.5505

Instituição Proponente: Universidade Federal de São Paulo - UNIFESP/EPM

Patrocinador Principal: Fundação de Amparo a Pesquisa de São Paulo ((FAPESP))

DADOS DO PARECER

Número do Parecer: 203.112

Data da Relatoria: 08/02/2013

Apresentação do Projeto:

Conforme Parecer CEP 192.249 de 1/2/2013

Objetivo da Pesquisa:

Conforme Parecer CEP 192.249 de 1/2/2013

Avaliação dos Riscos e Benefícios:

Conforme Parecer CEP 192.249 de 1/2/2013

Comentários e Considerações sobre a Pesquisa:

Conforme Parecer CEP 192.249 de 1/2/2013

Considerações sobre os Termos de apresentação obrigatória:

Conforme Parecer CEP 192.249 de 1/2/2013

Recomendações:

não se aplica

Conclusões ou Pendências e Lista de Inadequações:

Repostas apresentadas adequadamente.

Endereço: Rua Botucatu, 572 1º Andar Conj. 14
Bairro: VILA CLEMENTINO **CEP:** 04.023-061
UF: SP **Município:** SAO PAULO
Telefone: (11)5539-7162 **Fax:** (11)5571-1062 **E-mail:** cepunifesp@unifesp.br

UNIVERSIDADE FEDERAL DE SÃO PAULO - UNIFESP/ HOSPITAL SÃO PAULO

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

O colegiado acata o parecer do relator

SAO PAULO, 22 de Fevereiro de 2013

Assinador por:
maria del carmen janeiro perez
(Coordenador)

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Bairro: VILA CLEMENTINO **CEP:** 04.023-061
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Telefone: (11)5539-7162 **Fax:** (11)5571-1062 **E-mail:** cepunifesp@unifesp.br

7.2 Anexo 2: Aprovação do CONEP: Envio das amostras para o exterior

COMISSÃO NACIONAL DE ÉTICA EM PESQUISA

PARECER CONSUBSTANCIADO DA CONEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: INTERAÇÃO GENE-AMBIENTE EM INDIVÍDUOS EM ESTADO MENTAL DE RISCO PARA PSICOSE

Pesquisador: Rodrigo Affonseca Bressan

Área Temática: Genética Humana:
(Haverá envio para o exterior de material genético ou qualquer material biológico humano para obtenção de material genético, salvo nos casos em que houver cooperação com o Governo Brasileiro;);
(Haverá armazenamento de material biológico ou dados genéticos humanos no exterior e no País, quando de forma conveniada com instituições estrangeiras ou em instituições comerciais;);

Versão: 4

CAAE: 33148114.6.0000.5505

Instituição Proponente: Departamento de Psiquiatria

Patrocinador Principal: CENTRO DE ESTUDOS PAULISTA DE PSIQUIATRIA
Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.211.658

Apresentação do Projeto:
INTRODUÇÃO

O aparecimento da esquizofrenia é precedido por sintomas psicóticos isolados e uma fase prodrômica tardia com sintomas mais angustiantes e persistentes, chamado de Estado Mental de Risco (EMR). Porém, esses sintomas psicóticos isolados podem ocorrer em 10-20% da população saudável, e apenas uma fração desses indivíduos continuarão apresentando esses sintomas de forma persistente, enquadrando-se no grupo de maior risco de desenvolver a psicose. Da mesma forma, familiares de pacientes com esquizofrenia tem um risco aumentado de apresentar sintomas psicóticos atenuados, sendo que apenas uma parcela desses indivíduos desenvolverão esquizofrenia. Pessoas com sintomas prodrômicos já apresentam sintomas psicóticos persistentes e tem um risco aumentado para a esquizofrenia, com uma taxa média de conversão para a doença de 30%.Contudo ainda é muito difícil predizer com precisão quais indivíduos em risco para

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Bairro: Asa Norte **CEP:** 70.750-521
UF: DF **Município:** BRASILIA
Telefone: (61)3315-5878 **E-mail:** conep@saude.gov.br

COMISSÃO NACIONAL DE
ÉTICA EM PESQUISA

Continuação do Parecer: 1.211.658

Folha de Rosto	Folha_derosto_CONEP.doc	19:53:40		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_352098.pdf	06/07/2015 20:03:35		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_EUGEI_final_alteracoes_marcadas.doc	09/07/2015 11:07:14		Aceito
Outros	Resposta_itens_do_CONEP.doc	09/07/2015 11:08:07		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_352098.pdf	09/07/2015 11:09:09		Aceito
Projeto Detalhado / Brochura Investigador	EUGEI_traduzido_FINALv2_alteracoes_marcadas.doc	15/07/2015 12:01:43		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_352098.pdf	15/07/2015 12:02:40		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Sim

BRASILIA, 05 de Setembro de 2015

Assinado por:
Jorge Alves de Almeida Venancio
(Coordenador)

Endereço: SEPN 510 NORTE, BLOCO A 3º ANDAR, Edifício Ex-INAN - Unidade II - Ministério da Saúde
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UF: DF **Município:** BRASILIA
Telefone: (61)3315-5878 **E-mail:** conep@saude.gov.br

7.3 **Anexo 3: Menção honrosa em congresso**

UNIVERSIDADE FEDERAL DE SÃO PAULO
ESCOLA PAULISTA DE MEDICINA
Programa de Pós-Graduação em Biologia Estrutural e Funcional

UNIFESP
UNIVERSIDADE FEDERAL DE SÃO PAULO
1933

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA ESTRUTURAL E FUNCIONAL

Menção Honrosa

O Programa de Pós-Graduação em Biologia Estrutural e Funcional da UNIFESP/EPM", confere Menção Honrosa a MARCOS LEITE SANTORO com o trabalho intitulado "ANÁLISE DE EXPRESSÃO DE MRNA E MIRNA NA BUSCA DE POTENCIAIS MARCADORES DE RISCO E DE CONVERSÃO PARA A PSICOSE EM INDIVÍDUOS EM ESTADO MENTAL DE RISCO (EMR)", orientado pelo(a) Dra. Sintia Iole Nogueira Belangero por ter sido selecionados entre o melhor projeto de DOUTORADO para concorrerem ao Prêmio José Carlos Prates, no "XVI Congresso do Programa de Pós-Graduação em Biologia Estrutural e Funcional da UNIFESP/EPM", realizado nos dias 18 e 19 de novembro de 2013.

São Paulo, 19 de Novembro de 2013.

Profa. Dra. Janete Maria Cerutti
Coordenadora do Programa de Pós-Graduação em Biologia Estrutural e Funcional

Patrocínio: life technologies™

CITOGEM

mec lab
Comércio de Produtos e Equipamentos para Laboratório

7.4 **Anexo 4: Prêmio Travel Award em congresso**

XXII[nd] World Congress of Psychiatric Genetics

presents a

2014 Travel Award

to

Marcos Santoro

October 12 – 16, 2014 at the WCPG Meeting in Copenhagen, Denmark.

Thomas G. Schulze, M.D.
Secretary of the International Society of Psychiatric Genetics

7.5 **Anexo 5: Prêmio Travel Award em congresso**

Schizophrenia International Research Society

Marcos Leite Santoro

is hereby presented this certificate of recognition for receiving an

SIRS TRAVEL FELLOWSHIP

for the 2016 Biennial Conference

René Kahn M.D., Ph.D., President

Cynthia Shannon Weickert, PhD., Secretary

7.6 Anexo 6: *Scripts* de R modificados ou adaptados pelo aluno para a análise dos artigos 3 e 4

7.6.1 Pré-processamento dos *microarrays* de genotipagem e análise GWAS pelo PLINK

Pré-processamento Genotipagem:

- Utilizei o *genome studio* seguindo o passo-a-passo do Stephan Newhouse em: https://confluence.brc.iop.kcl.ac.uk:8493/display/PUB/Production+Version%3A+Illumina+Exome+Chip+SOP+v1.4;jsessionid=D2D7B21326B220358E631D60445857B4
- Manifest file: Marcos_infinium-hts-PsychChi ... amplesheet181215_notepad.txt

Alterações no passo-a-passo original devido ao meu tamanho de amostra:

- *clustersep* < 0.36
- *het excess* até 0.2
- *AA teta mean* = removi os SNPs zerados, reordenei por *teta mean* e depois por *freq AA* excluindo aqueles SNPs maiores que 0,35 e com *frreq*=100% de AA. Por fim, voltei a ordenar por *teta mean* e, utilizando o parâmetro de *dev,* olhei com cuidado aqueles SNPs maiores que 0.04 e menores que 0.25
- Para *BB teta mean*, exclui os SNPs até 0.67 com BB 100%
- Não exclui nenhum SNP em desequilíbrio de Hardy-Weinberg nesse momento
- Não apliquei a correção de *minor allele frequence* nesse momento
- Eu não rodei o zCall ou GenCal após estes passos (passos importantes para encontrar CNVs ou outros polimorfismos).

PLINK:

#Map/ped para bed:

plink --file BRFEP_3012_01 --make-bed --out BRFEP

#QC:

plink --bfile BRFEP --maf 0.01 --geno 0.1 --hwe 0.00001 --make-bed --out BRFEP_QC

plink --bfile BRFEP_QC --mind 0.01 --make-bed --out BRFEP_QC

#Checar se continua com algum missing:

plink --bfile BRFEP_QC --missing --out missing

sort -k 5 -gr missing.lmiss | head

#aplicar o Scrip de R para a imputação e preparação da amostra para o escore poligênico

Estudo de associação (adaptado de Diego Mazzotti e Jonathan Coleman):

PRUNE:

./plink --bfile BRFEP_QC --indep-pairwise 1500 150 0.2 --out PRUNE_LD_ONE

./plink2 --bfile BRFEP_QC --extract PRUNE_LD_ONE.prune.in --make-bed --out BRFEP_PRUNE_LD_two

wc BRFEP_PRUNE_LD_two.bim

awk –f highLDregions4bim_b37.awk BRFEP_PRUNE_LD_two.bim > highLDexcludes

awk '($1 < 1) || ($1 > 22) {print $2}' BRFEP_PRUNE_LD_two.bim > autosomeexcludes

head autosomeexcludes

./plink2 --bfile BRFEP_PRUNE_LD_two --exclude autosomeexcludes --make-bed --out BRFEP_PRUNE_LD_3

./plink2 --bfile BRFEP_PRUNE_LD_3 --update-ids update_IDS --make-bed --out BRFEP_PRUNE_LD_31

./plink2 --bfile BRFEP_PRUNE_LD_31 --pheno pheno2 --make-bed --out BRFEP_PRUNE_LD_4

```
./plink2 --bfile BRFEP_PRUNE_LD_two --check-sex ycount --out BRFEP_sex_check

./plink2 --bfile BRFEP_PRUNE_LD_4 --genome --make-bed --out BRFEP_IBD

awk '$10 >= 0.1875 {print $1, $2}' BRFEP_IBD.genome > BRFEP_IBD_outliers.txt

./plink2 --bfile BRFEP_PRUNE_LD_4  --assoc --adjust --out assoc_bruta

./plink2 --bfile BRFEP_PRUNE_LD_4  --read-genome BRFEP_IBD --cluster --mds-plot 10 --out MDS10

sudo R

dados_MDS <- read.table("MDS10.mds", header=T)

head(dados_MDS10)

library(rgl)

plot3d(dados_MDS$C1, dados_MDS$C2, dados_MDS$C3, type="p", col="blue")

./plink2 --bfile BRFEP_PRUNE_LD_4 --logistic --adjust --covar MDS10.mds --covar-number 2-10 --out assoc_logistic

./plink2 --bfile BRFEP_PRUNE_LD_4 --logistic --adjust --covar MDS10.mds --covar-number 2-11 --qq-plot

./plink2 --bfile BRFEP_PRUNE_LD_4 --logistic --adjust --covar MDS10.mds --covar-number 2-5 --qq-plot
```

7.6.2 Pré-processamento para realizar o escore poligênico de risco utilizando o PRSice e para realizar a imputação dos dados.

```
# setar onde
setwd("~/Documents/Arrays_PEP/Genotyping/PLINK_050116_0226_zerado/")
# leia o bim, mude o nome do arquivo neh...
bim <- read.table("FEP_QCed.bim", header = F)
# quero todo mundo exceto quem for duplicado na coluna 4
bim_teste <-bim[!duplicated(bim[,4]), ]
#bim_teste2 <- bim[!duplicated(bim[,2]), ]
# só quero a coluna que restou de SNPs
SNPs_incluidos <- bim_teste[,2]
# escrever a tabela de SNPs incluidos
write.table(SNPs_incluidos, file="SNPs_sem_dup.txt", row.names=FALSE, quote = FALSE, col.names=F)
#bim <- read.table("INPD_chr22imp_QCpass_final_original.bim", header = F)
#V7 <- c(1:nrow(bim_teste))
#bim2 <- cbind(bim_teste, V7)
ins <- bim_teste[((bim_teste$V5=="A" | bim_teste$V5=="T" | bim_teste$V5=="G" | bim_teste$V5=="C") & (bim_teste$V6=="A"
|
bim_teste$V6=="T" | bim_teste$V6=="G" | bim_teste$V6=="C")),]
outs <- bim_teste[!((bim_teste$V5=="A" | bim_teste$V5=="T" | bim_teste$V5=="G" | bim_teste$V5=="C") &
(bim_teste$V6=="A" | bim_teste$V6=="T" | bim_teste$V6=="G" | bim_teste$V6=="C")),]
#outs$V2 <- sub("^", "chr", outs$V2)
SNPsOut <- outs$V2
write.table(SNPsOut, file="SNPsOut.txt", row.names=FALSE, quote = FALSE)
#marcado <- rbind (ins, outs)
#bim_ordenado <- marcado[order(marcado$V7),]
#bim_final <- bim_ordenado[ , c("V1","V2", "V3","V4","V5","V6")]
#write.table(bim_final, file="INPD_chr22imp_QCpass_final.bim", dec=".", row.names=FALSE,sep="\t", quote = FALSE,
col.names=FALSE)
# SNP_ID Old_A1 Old_A2 New_A1 New_A2
setwd("/home/marcossantoro/Documents/Arrays_PEP/Genotyping/GenomeStudio_manifest_and_egt/")
manifest <- read.csv("PsychChip_v1-1_15073391_C.csv", header=T, skip=7)
head(manifest)
#PsychArray_manifest <- read.csv("InfiniumPsychArray-24v1-1_A1.csv", header=T, skip=7)
#head(manifest)
setwd("/home/marcossantoro/Documents/Arrays_PEP/Genotyping/PLINK_050116_0226_zerado/")
#bim <- read.table("FEP_QCed.bim", header=F)
head(ins)
bim <- ins
#bim <- bim[order(bim$V2),]
QCed <- bim$V2
manifesta2 <- manifest[manifest$Name %in% QCed,]
#PsyArr_manifesta2 <- PsychArray_manifest[PsychArray_manifest$Name %in% QCed,]
#SNPs_PsyChi <- manifesta2$Name
#Sem_Strand <- QCed[!QCed %in% PsyArr_manifesta2$Name]
#Sem_Strand <- as.data.frame(Sem_Strand)
#SNPs_comStrand <- PsyArr_manifesta2$Name
#SNPs_comStrand <- as.data.frame(SNPs_comStrand)
#write.table(SNPs_comStrand, file="SNPs_comStrand.txt", sep=" ", col.names=F, row.names=F, quote=F)
#write.table(Sem_Strand, file="Sem_strand.txt", sep=" ", col.names=F, row.names=F, quote=F)
# teste <- manifesta2[grep("rs", manifesta2$Name), ]
# Ir para o plink excluir esses FDPs
#NewBim <- read.table("PSYArray.bim", header=F)
#head(NewBim)
#NewBim <- NewBim[order(NewBim$V2),]
#PsyArr_manifesta2 <- PsyArr_manifesta2[order(PsyArr_manifesta2$Name),]
#head(NewBim)
#head(PsyArr_manifesta2)
#bim_manifest <- cbind(NewBim,PsyArr_manifesta2)
#head(bim_manifest)
#tail(bim_manifest)
#nega <- c("-")
#flippers <- bim_manifest[bim_manifest$RefStrand %in% nega,]
#flippers_final <- flippers$Name
#write.table(flippers_final, file="flippers.txt", sep=" ", col.names=T, row.names=F, quote=F)
#bim_manifest <- bim_manifest[,c("V2","V5", "V6", "SNP")]
#head(bim_manifest)
#bim_manifest$SNP = gsub("[[]", "", bim_manifest$SNP)
#bim_manifest$SNP = gsub("[]]", "", bim_manifest$SNP)
#library(splitstackshape)
```

```
#New_alleles <- cSplit(bim_manifest, "SNP", "/")
#colnames(New_alleles) <- c("SNP_ID", "Old_A1", "Old_A2", "New_A1", "New_A2")
#head(New_alleles)
#write.table(New_alleles, file="New_alleles.txt", sep=" ", col.names=T, row.names=F, quote=F)
cabeca <- c("V1", "SNP", "FID", "V2", "A1","A2")
names(bim) <- cabeca
#binzinho <- bim[, c("V2","V5")]
#write.table(binzinho, file="a2.txt", sep=" ", col.names=F, row.names=F, quote=F)
HRC <- read.table("xaa", header=F)
head(HRC)
gc()
abrolhos <- merge(bim, HRC, by=c("V1","V2"))
HRC <- read.table("xab", header=F)
head(HRC)
gc()
abrolhos1 <- merge(bim, HRC, by=c("V1","V2"))
HRC <- read.table("xac", header=F)
head(HRC)
gc()
abrolhos2 <- merge(bim, HRC, by=c("V1","V2"))
HRC <- read.table("xad", header=F)
head(HRC)
gc()
abrolhos3 <- merge(bim, HRC, by=c("V1","V2"))
HRC <- read.table("xae", header=F)
head(HRC)
gc()
abrolhos4 <- merge(bim, HRC, by=c("V1","V2"))
HRC <- read.table("xaf", header=F)
head(HRC)
gc()
abrolhos5 <- merge(bim, HRC, by=c("V1","V2"))
abrolhos_fim <- rbind(abrolhos,abrolhos1,abrolhos2,abrolhos3,abrolhos4,abrolhos5)
REF <-abrolhos_fim[!duplicated(abrolhos_fim[,3]), ]
REF_fim <- REF[,c("SNP","V4")]
#REF_fu <- REF[,c("SNP")]
#REF_fu <- as.data.frame(REF_fu)
write.table(REF_fim, file="REF.txt", sep=" ", col.names=F, row.names=F, quote=F)
#write.table(REF_fu, file="REF_inc.txt", sep=" ", col.names=F, row.names=F, quote=F)
rm (abrolhos, abrolhos1,abrolhos2,abrolhos3,abrolhos4,abrolhos5,HRC,bim2,bim_teste,bim_teste2)
gc()
REF_fim <- read.table("REF.txt", header=F)
bim_limpo <- read.table("FEP_QCed_semdup_semindel_flipado.bim")
rownames(bim_limpo) <- bim_limpo$V2
ficam <- REF_fim$V1
saem <- bim_limpo[!(bim_limpo$V2 %in% REF_fim$V1),]
write.table(saem$V2, file="nao_imputados.txt", sep=" ", col.names=F, row.names=F, quote=F)
# ir para o plink
Jiraiya <- read.table("detectar_flips.log", header=F, skip=17, fill=T)
Jiraiya2 <- Jiraiya[1:167489,]
tail(Jiraiya2)
Jiraiyito <- Jiraiya2$V8
write.table(Jiraiyito, file="Jirai-ya.txt", sep=" ", col.names=F, row.names=F, quote=F)
# flipar no plink
```

7.6.3 Associação entre variáveis clínicas e o PRS

```
#Lendo bancos
library(reshape)
library(plyr)
##############################
# Preparar banco com variáveis clínicas e resultado do Escore Poligênico de risco (PRS)
##########################
# Ler o último banco clínico
PEPclinico <- read.csv("~/Downloads/Banco PEP clinico genetico 07.08.2015 Renan_panssatual-2.csv",
                       header=TRUE, stringsAsFactors=FALSE, fileEncoding="latin1")
#Quem foi genotipado?
PSY <- read.table("~/Documents/PepGeno.txt")

row.names(PEPclinico) <- as.character(PEPclinico$ID)

#Só me interessa aqueles que foram genotipados
PEPclinico_PSY <- PEPclinico[as.character(PSY$V1),]

#Chamar os componentes principais
## NOMHC
##PCs <- read.table("~/Downloads/FEP_maf001_nomhc_ANCESTRY_INFORMATIVE_DIMENSIONS.mds", header = T)
## MHC
PCs <- read.table("~/Downloads/re/ANCESTRY_INFORMATIVE_DIMENSIONS.mds", header = T)

#Chamar a tabela de escore
##NOMHC
#escore <- read.table("~/Downloads/FEP_maf001_nomhc_SCORES_AT_ALL_THRESHOLDS.txt", header = T)

##MHC
#escore <- read.table("~/Documents/PLINK_050116_0226_zerado/FEP_FINAL_190116_SCORES_AT_ALL_THRESHOLDS.txt", header = T)
escore <- read.table("~/Downloads/re/FEP_maf001_MHC_1909_SCORES_AT_ALL_THRESHOLDS.txt", header = T)
#no imput
#escore <- read.table("~/Documents/PLINK_050116_0226_zerado/FEP_semImp_SCORES_AT_ALL_THRESHOLDS.txt", header = T)

# preparar para o merge e selecionar quais escores manter
escore$ID <- PCs$IID
PCs$ID <- PCs$IID
BestScores <- c("ID","pT_0.0001","pT_0.001","pT_0.01","pT_0.0112","pT_0.05","pT_0.1","pT_0.2","pT_0.3","pT_0.4","pT_0.5")
Scores <- escore[,BestScores]

#merge do escore com os componentes principais
Scores <- join_all(list(Scores,PCs), by = "ID", type = "full")
#arrumar o ID, no banco clínico o follow-up é distinguido pelas colunas
Scores$ID <- gsub("PW", "P", Scores$ID)
```

```
Scores$ID <- gsub("P", "P|", Scores$ID)
# Aquele mesmo erro de sempre... alguns bancos está como P10 e outros P010
Scores2 <- transform(Scores, ID = colsplit(ID, split = "\\|", names = c('a', 'b')))
Scores2$ID$b <- sprintf("%03s", Scores2$ID$b)
Scores2$ID <- paste(Scores2$ID$a,Scores2$ID$b, sep = "")

#confirmar se os dois são iguais e aplicar o merge
Scores2 <- Scores2[order(Scores2$ID),]
identical(PEPclinico_PSY$ID,Scores2$ID)
PEP_mergePSY <- join_all(list(Scores2,PEPclinico_PSY), by = "ID", type = "full")

# Remover os Frangos da Rocha; escalas psiquiátricas foram aplicadas inadequadamente
frangos <- PEP_mergePSY$ID %in% c("P035","P091","P154")
PEP_mergePSY <- PEP_mergePSY[!frangos,]

#Novas variáveis; Pontuação total da PANSS para sintoamas positivos, negativos, gerais; Variáveis de resposta
# delta entre baseline - follow-up
PEP_mergePSY$PanssNEG1_TOT <- PEP_mergePSY$N1_1 +PEP_mergePSY$N2_1 +PEP_mergePSY$N3_1
+PEP_mergePSY$N4_1 +PEP_mergePSY$N5_1 +PEP_mergePSY$N6_1 +PEP_mergePSY$N7_1 -7
PEP_mergePSY$PanssNEG2_TOT <- PEP_mergePSY$N1_2 +PEP_mergePSY$N2_2 +PEP_mergePSY$N3_2
+PEP_mergePSY$N4_2 +PEP_mergePSY$N5_2 +PEP_mergePSY$N6_2 +PEP_mergePSY$N7_2 -7
PEP_mergePSY$PanssPOS1_TOT <- PEP_mergePSY$P1_1 +PEP_mergePSY$P2_1 +PEP_mergePSY$P3_1
+PEP_mergePSY$P4_1 +PEP_mergePSY$P5_1 +PEP_mergePSY$P6_1 +PEP_mergePSY$P7_1 -7
PEP_mergePSY$PanssPOS2_TOT <- PEP_mergePSY$P1_2 +PEP_mergePSY$P2_2 +PEP_mergePSY$P3_2
+PEP_mergePSY$P4_2 +PEP_mergePSY$P5_2 +PEP_mergePSY$P6_2 +PEP_mergePSY$P7_2 -7
PEP_mergePSY$PanssG_TOT1 <- PEP_mergePSY$G1_1 +PEP_mergePSY$G2_1 +PEP_mergePSY$G3_1
+PEP_mergePSY$G4_1 +PEP_mergePSY$G5_1 +PEP_mergePSY$G6_1 +PEP_mergePSY$G7_1 +
PEP_mergePSY$G8_1 +PEP_mergePSY$G9_1 +PEP_mergePSY$G10_1 +PEP_mergePSY$G11_1
+PEP_mergePSY$G12_1 +PEP_mergePSY$G13_1 +PEP_mergePSY$G14_1+PEP_mergePSY$G15_1
+PEP_mergePSY$G16_1 -16
PEP_mergePSY$PanssG_TOT2 <- PEP_mergePSY$G1_2 +PEP_mergePSY$G2_2 +PEP_mergePSY$G3_2
+PEP_mergePSY$G4_2 +PEP_mergePSY$G5_2 +PEP_mergePSY$G6_2 +PEP_mergePSY$G7_2 +
PEP_mergePSY$G8_2 +PEP_mergePSY$G9_2 +PEP_mergePSY$G10_2 +PEP_mergePSY$G11_2
+PEP_mergePSY$G12_2 +PEP_mergePSY$G13_2 +PEP_mergePSY$G14_2+PEP_mergePSY$G15_2
+PEP_mergePSY$G16_2 -16

PEP_mergePSY$Resposta_simples <- PEP_mergePSY$Pansstot_1 - 30 - PEP_mergePSY$Pansstot_2 - 30
PEP_mergePSY$Resposta_completa <- ((PEP_mergePSY$Pansstot_1 -30) - (PEP_mergePSY$Pansstot_2 - 30))*100 /
(PEP_mergePSY$Pansstot_1 - 30)
PEP_mergePSY$Resposta_NEG_simples <- PEP_mergePSY$PanssNEG1_TOT - PEP_mergePSY$PanssNEG2_TOT
PEP_mergePSY$Resposta_NEG_completa <- (PEP_mergePSY$PanssNEG1_TOT -
PEP_mergePSY$PanssNEG2_TOT)*100/PEP_mergePSY$PanssNEG1_TOT
PEP_mergePSY$Resposta_POS_simples <- PEP_mergePSY$PanssPOS1_TOT - PEP_mergePSY$PanssPOS2_TOT
PEP_mergePSY$Resposta_POS_completa <- ((PEP_mergePSY$PanssPOS1_TOT) -
(PEP_mergePSY$PanssPOS2_TOT))*100/PEP_mergePSY$PanssPOS1_TOT

PEP_mergePSY$Respondedor_new <- ifelse(PEP_mergePSY$Resposta_completa >50, 0, 1)
PEP_mergePSY$panss_depa2_teste <- (PEP_mergePSY$G2_2 + PEP_mergePSY$G3_2 +PEP_mergePSY$G4_2 +
PEP_mergePSY$G6_2)*9/4
```

```
PEP_mergePSY$panss_neg_tot_simples <- PEP_mergePSY$panss_neg1 - PEP_mergePSY$panss_neg2 + 33
PEP_mergePSY$panss_pos_tot_simples <- PEP_mergePSY$panss_pos1 - PEP_mergePSY$panss_pos2 + 5
PEP_mergePSY$panss_dis_tot_simples <- PEP_mergePSY$panss_dis1 - PEP_mergePSY$panss_dis2 + 11
PEP_mergePSY$panss_depa_tot_simples <- PEP_mergePSY$panss_depa1 - PEP_mergePSY$panss_depa2 + 24
PEP_mergePSY$panss_exc_tot_simples <- PEP_mergePSY$panss_exc1 - PEP_mergePSY$panss_exc2 + 12
PEP_mergePSY$calgarytot_simples <- PEP_mergePSY$calgarytot_1 - PEP_mergePSY$calgarytot_2 +12

# Banco de dados final
write.csv(PEP_mergePSY, file="PEP_mergePSY280716.csv")

###################
#Análise de associação entre o PRS e as variáveis clínicas
###################
# Só me interessam os pacientes para fazer a análise PRS x variáveis clínicas
pacientes <- PEP_mergePSY[60:119,]

# variáveis selecionadas (criar um método menos manual...)
names(pacientes)
n <- 950
vars <- c(78:79,302:359,423,424,633:667,672, 673,906:952)
vars_kendall <- c(358,666,423, 424, 672,673,311,619,324,632,340,648,332,640, 924:943,945:952)
vars_poisson <- c(358,666,423, 424, 672,673,311,619,324,632,340,648,332,640, 924:939,948:953)

#minhas variáveis dependentes
var_deps <- pacientes[,vars_kendall]
var_deps_kendall <- pacientes[,vars_kendall]
var_deps_poisson <- pacientes[,vars_poisson]

#scores_inFEP <- pacientes[,2:11]
#mods <- apply(var_deps, 2 , FUN = function(x){ lm( x ~ pacientes$pT_0.0112)} )

# Número de observações para cada variável clínica
N_amostral <- as.data.frame(apply(var_deps_poisson, 2 , FUN = function(x){ length(x[!is.na(x)])} ))

#regressao linear (não foi utilizada; variáveis clínicas são ordinais, optamos pela regressão de Poisson)
mods2 <- apply(var_deps, 2 , FUN = function(x){ lm( x ~ pacientes$pT_0.0112)} ) # sem covariável
mods2 <- apply(var_deps, 2 , FUN = function(x){ lm( x ~ pacientes$pT_0.0112 + pacientes$C1+ pacientes$C2+ pacientes$C3
+ pacientes$C4)}) # 4 covars
mods2 <- apply(var_deps, 2 , FUN = function(x){ lm( x ~ pacientes$pT_0.0112 + pacientes$C1+ pacientes$C2)} ) # covars
#pvals_LM <- sapply( mods2 , function(x)( summary(x)$p.value[,3] ))

#regressao poisson (****estatística utilizada para o artigo 3****)
mods3 <- apply(var_deps_poisson, 2 , FUN = function(x){ glm( formula = x ~ pT_0.0112 + C1 + C2, data = pacientes, family =
poisson)}) # 2 covars
mods3 <- apply(var_deps_poisson, 2 , FUN = function(x){ glm( formula = x ~ pT_0.0112, data = pacientes, family = poisson)} )
# sem covars
mods3 <- apply(var_deps_poisson, 2 , FUN = function(x){ glm( formula = x ~ pT_0.0112 + C1 + C2 + C3 + C4, data =
pacientes, family = poisson)}) #4 covars
```

```
# Prepara tabela com o B, número de observações e valor de p para cada variável
pvals_poisson <- as.data.frame(t(sapply( mods3 , function(x)(summary(x)$coefficients[,4]))))
estimate_poisson <- as.data.frame(t(sapply( mods3 , function(x)(summary(x)$coefficients[,1]))))
tabela_final_poisson <- as.data.frame(cbind(estimate_poisson$pT_0.0112,pvals_poisson$pT_0.0112, N_amostral))
# Salvar com o nome correto
write.table(tabela_final_poisson, file = "poisson_results_MHC_C4_1909.txt", row.names = T, col.names = T)

#Visualizar os resultados no R
tabela_poisson <- as.data.frame(round(pvals_poisson, digits = 6))
#tabela_poisson <- as.data.frame(pvals_poisson)
bacanas_poisson <- subset(tabela_poisson, tabela_poisson$pT_0.0112 <= 0.05)
View(bacanas_poisson)

# Testar se respondedores x não respondedores tem diferença de média para o PRS
t.test(pacientes$pT_0.0112 ~ pacientes$Respondedor_new)

#kendall correlation (estatística não utilizada no artigo); Optei pela correlação de Kendal por ela lidar bem com empates
# e com variáveis ordinais
mods <- apply(var_deps_kendall, 2 , FUN = function(x){ cor.test( x, pacientes$pT_0.0112, method = "kendall")} )

# kendal correlation: tabela final e visualização dos resultados no R
pvals_kendall <- sapply( mods , function(x)( x$p.value))
estimate_kendall <- sapply( mods , function(x)( x$estimate))
statistic_kendall <- sapply( mods , function(x)( x$statistic))
tabela_final_kendall <- as.data.frame(cbind(statistic_kendall,estimate_kendall,pvals_kendall, N_amostral))
write.table(tabela_final_kendall, file = "kendall_results_nomhc.txt", row.names = T, col.names = T)
tabela <- as.data.frame(t(pvals_kendall))
tabela <- as.data.frame(pvals_kendall)
bacanas <- subset(tabela, tabela$pvals_kendall <= 0.05)
View(bacanas)

##########
#FIM
##########
```

7.6.4 Pré-processamento dos *microarrays* de expressão (adaptado)

https://github.com/snewhouse/BRC_MH_Bioinformatics/tree/master/Illumina_expression_workflow

7.6.5 Análise de probes diferencialmente expressas entre os grupos

```
#biblios que eu preciso
library(oligo)
library(limma)
library(bioDist)
library(annotate)
library(hugene10sttranscriptcluster.db)
library(lumi)
library(xtable)

############
# Análise para identificar probes diferencialmente expressas entre Caso x Controle (CACO)
# e entre Follow-up x Casos (FOCA)
############

setwd("~/Documents/FEP_finalissimo_lumi_processing_12_11_2015_1447336480/eset_final/")

#Chamar os resultado do pipleine "foca2_corrigido.R" (adaptado de S.NEWHOUSE no github)
load("FEP_finalissimo.eset_final.RData")
eset_final

# Banco atualizado com as caractrísticas da amostra
pDat <- read.table("pDat_EXP_final2804.txt", header=T, sep="\t", dec=".")

#ESET PARA CASO X CONTROLE
caco <- pDat[pDat$CACO==0,]
caquito <- as.character(caco$sampleID)
eset_caco <- eset_final[,caquito]
dim(eset_caco)
#eset_caco2 <- eset_caco[-grep("X", eset_caco@featureData@data$CHROMOSOME),]
#eset_caco3 <- eset_caco2[-grep("Y", eset_caco2@featureData@data$CHROMOSOME),]

#ESET PARA BASELINE X FOLLOW-UP DE DOIS MESES (FOCA)
foca <- pDat[pDat$FOCA==0,]
eset_foca <- eset_final[,as.character(foca$sampleID)]
foco <- foca[duplicated(foca$Pareado),]
precisao <- foca[!duplicated(foca$Pareado),]
saem <- as.character(precisao$Pareado[!precisao$Pareado %in% ficam])
ficam <- as.character(foco$Pareado)

eset_PAREfoca <- eset_foca[,pData(eset_foca)$ORIGINAL_ID %in% ficam]
ficam_pdat <- row.names(pData(eset_PAREfoca))
```

```
pDat_foca <- pDat[pDat$sampleID %in% ficam_pdat,]
dim(eset_PAREfoca)
dim(pDat_foca)
#eset_PAREfoca2 <- eset_PAREfoca[-grep("X", eset_PAREfoca@featureData@data$CHROMOSOME),]
#eset_PAREfoca3<-eset_PAREfoca2[-grep("Y", eset_PAREfoca2@featureData@data$CHROMOSOME),]
#foica <- pDat[pDat$FOiCA==0,]
#PAREfoquita <- duplicated(foca$Pareado)

# Variáveis que vou utilizar e que estão no meu pDat
grupos <- factor(pDat$PHENOTYPE, levels=c("CONTROL", "CASE"))
sex <- factor(pDat$SEX, levels=c("MALE", "FEMALE"))
age <- pDat$AGE
fuma <- factor(pDat$SMOKE, levels=c("1", "2")) #só se era fumante no momento da coleta
fumou <- factor(pDat$SMOKED, levels=c("1", "2")) #EVER SMOKED
C1 <- pDat$C1  # Componentes principais da genotipagem
C2 <- pDat$C2
C3 <- pDat$C3
C4 <- pDat$C4
C5 <- pDat$C5
C6 <- pDat$C6
C7 <- pDat$C7
C8 <- pDat$C8
C9 <- pDat$C9
C10 <- pDat$C10
C11 <- pDat$C11
C12 <- pDat$C12
C13 <- pDat$C13
C14 <- pDat$C14
C15 <- pDat$C15
C16 <- pDat$C16
C17 <- pDat$C17
C18 <- pDat$C18
C19 <- pDat$C19
C20 <- pDat$C20

B <- pDat$Ba # porcentagem dos tipos celulares para cada indivíduo (CellMix.R)
eri <- pDat$eri
mono <- pDat$monoa
#megak <- pDat$megak
NK2 <- pDat$NKa
gran <- pDat$grana
cd4 <- pDat$cd4a
cd8 <- pDat$cd8a
neutro <- pDat$NEUTRO
NK <- pDat$NK
monogb <- pDat$MONO
#cor <- pDat$COR
tc <- pDat$TC
tc_act <- pDat$TC_ACT
```

```
igg <- pDat$IGG
igm <- pDat$IGM
#dc <- pDat$DC_ACT
EXPC1 <- pDat$EXPC1 # Componentes principais da expressão gênica
EXPC2 <- pDat$EXPC2
EXPC3 <- pDat$EXPC3
EXPC4 <- pDat$EXPC4

####################################
# quais serão as minhas covariaveis?
####################################
#GedBlood
# Este foi utilizado o artigo 4
design <- model.matrix(~grupos+sex+age+fuma+C1+C2+C3+C4+
                       neutro+NK+monogb+igg+igm+tc+tc_act)
#design <- model.matrix(~grupos+sex+age+fuma+C1+C2+C3+C4+C5+C6+C7+C8+C9+
 #                        C10+C11+C12+C13+C14+C15+C16+C17+C18+C19+C20+neutro
  #                      +NK+monogb+igg+igm+tc+tc_act)
#design <- model.matrix(~grupos+sex+age+fuma+C1+C2+C3+C4+EXPC1+EXPC2)
#Ged
#design <- model.matrix(~grupos+sex+age+fuma+C1+C2+C3+C4+B+eri+NK2+gran+cd4+cd8)
#No blood
#design <- model.matrix(~grupos+sex+age+fuma+C1+C2+C3+C4)
#design <- model.matrix(~grupos+sex+age+fuma+cor)
#design <- model.matrix(~0+grupos)

#Fit
fit <- lmFit(eset_caco, design)
fit2 <- contrasts.fit(fit, coef="gruposCASE")
fit3 <- eBayes(fit2)
topTable(fit3, coef="gruposCASE", adjust="bonferroni")
results <- decideTests(fit2)
results_bonferroni <- decideTests(fit2, adjust="bonferroni")

# Visualizar resultados com FDR e com bonferroni
#FDR
vennDiagram(results)
vennDiagram(results, include="up")
vennDiagram(results, include="down")
#Bonferroni
vennDiagram(results_bonferroni)
vennDiagram(results_bonferroni, include="up")
vennDiagram(results_bonferroni, include="down")

#hora de organizar os meus resultados
resultados_CACO <- topTable(fit3, coef="gruposCASE", adjust.method="bonferroni",
                            number=Inf)
# só isso que me importa:
resultados_CACO <- resultados_CACO[,c("SYMBOL","TRANSCRIPT", "ACCESSION","REFSEQ_ID"
```

```
                    ,"ENTREZ_GENE_ID", "PROBE_ID","DEFINITION","CHROMOSOME"
                    ,"PROBE_CHR_ORIENTATION","PROBE_COORDINATES",
                    "logFC","P.Value","adj.P.Val","B")]

#volcano plot com o resultado de eBayes
volcanoplot(fit3, coef="gruposCASE", highlight=20, names=fit3$genes$SYMBOL)

#Só os top 100
#top1000_CACO <- topTable(fit3, coef="gruposCASE", adjust.method="bonferroni", number=1000)
top100_CACO <- topTable(fit3, coef="gruposCASE", adjust.method="bonferroni", number=100)
top100_CACO <- top100_CACO[,c("SYMBOL","TRANSCRIPT", "ACCESSION","REFSEQ_ID","ENTREZ_GENE_ID"
                    , "PROBE_ID","DEFINITION","CHROMOSOME","PROBE_CHR_ORIENTATION"
                    ,"PROBE_COORDINATES","logFC","P.Value","adj.P.Val","B")]


#############
# ATENÇÃO!!! Checar o nome que vai salvar
#############
#Salvando:
write.table(resultados_CACO, file="Resultados_CACO_030516semsangue4C.txt",sep="\t",
        row.names=F,quote=FALSE)
write.table(top100_CACO,file="top100_CACO_030516semsangue4C.txt", sep="\t", row.names=F,
        quote=FALSE)

#####################################################################
#FIM DO CACO
#####################################################################
########################
# PREPARAR PARA O WGCNA OU LEARNING MACHINE
########################
design <- model.matrix(~grupos-grupos+sex+age+fuma+neutro+NK+monogb+igg+igm+tc+tc_act)
Fit1 <- lmFit(eset_caco, design)
correctedValues <- residuals.MArrayLM(Fit1, eset_caco)
write.table(correctedValues, file="Corrigido_MDS_cell_020216_GB.txt",sep=" ", row.names=F,
        quote=FALSE)

#####################################################################
#PAREADO -> FOCA
#####################################################################
pDat_foca$PHENOTYPE <- gsub("8","",pDat_foca$PHENOTYPE)
PAREgrupos <- factor(pDat_foca$PHENOTYPE, levels=c("CASE","WFOLLOWUP"))
ID_PEP <- factor(pDat_foca$Pareado)

sex <- factor(pDat_foca$SEX, levels=c("MALE", "FEMALE"))
age <- pDat_foca$AGE
fuma <- factor(pDat_foca$SMOKE, levels=c("1", "2")) #só se era fumante no momento da coleta
fumou <- factor(pDat_foca$SMOKED, levels=c("1", "2")) #EVER SMOKED,
C1 <- pDat_foca$C1
C2 <- pDat_foca$C2
```

```
C3 <- pDat_foca$C3
C4 <- pDat_foca$C4
C5 <- pDat_foca$C5
C6 <- pDat_foca$C6
C7 <- pDat_foca$C7
C8 <- pDat_foca$C8
C9 <- pDat_foca$C9
C10 <- pDat_foca$C10
C11 <- pDat_foca$C11
C12 <- pDat_foca$C12
C13 <- pDat_foca$C13
C14 <- pDat_foca$C14
C15 <- pDat_foca$C15
C16 <- pDat_foca$C16
C17 <- pDat_foca$C17
C18 <- pDat_foca$C18
C19 <- pDat_foca$C19
C20 <- pDat_foca$C20

B <- pDat_foca$Ba
eri <- pDat_foca$eri
mono <- pDat_foca$monoa
#megak <- pDat_foca$megak
NK2 <- pDat_foca$NKa
gran <- pDat_foca$grana
cd4 <- pDat_foca$cd4a
cd8 <- pDat_foca$cd8a
neutro <- pDat_foca$NEUTRO
NK <- pDat_foca$NK
monogb <- pDat_foca$MONO
#cor <- pDat_foca$COR
tc <- pDat_foca$TC
tc_act <- pDat_foca$TC_ACT
igg <- pDat_foca$IGG
igm <- pDat_foca$IGM
#dc <- pDat_foca$DC_ACT
EXPC1 <- pDat_foca$EXPC1
EXPC2 <- pDat_foca$EXPC2
EXPC3 <- pDat_foca$EXPC3
EXPC4 <- pDat_foca$EXPC4

#GedBlood
PAREdesign <- model.matrix(~ID_PEP+PAREgrupos+neutro+NK+monogb+igg+igm+tc+tc_act)
PAREdesign <- model.matrix(~ID_PEP+PAREgrupos)
#Ged
PAREdesign <- model.matrix(~PAREgrupos+ID_PEP+B+eri+mono+NK+cd4+gran+cd8)
PAREdesign <- model.matrix(~PAREgrupos+ID_PEP+B+eri+mono+NK+cd4+gran+cd8)
#No blood
#design <- model.matrix(~grupos+sex+age+fuma+C1+C2+C3+C4+C5+C6+C7+C8+C9+C10)
```

```
#design <- model.matrix(~grupos+sex+age+fuma+cor)
#design <- model.matrix(~0+grupos)

PAREfit <- lmFit(eset_PAREfoca3, PAREdesign)

PAREfit2 <- eBayes(PAREfit)
PAREresults <- decideTests(PAREfit2)
PAREresults2 <- decideTests(PAREfit2, adjust="bonferroni")

PAREresultados <- topTable(PAREfit2,coef="PAREgruposWFOLLOWUP", adjust="bonferroni",
                  number=Inf)
PAREresultados <- PAREresultados[,c("SYMBOL","TRANSCRIPT", "ACCESSION","REFSEQ_ID",
                        "ENTREZ_GENE_ID", "PROBE_ID","CHROMOSOME",
                        "PROBE_CHR_ORIENTATION","PROBE_COORDINATES",
                        "logFC","P.Value","adj.P.Val","B")]

volcanoplot(PAREfit2, coef="PAREgruposWFOLLOWUP", highlight=10, names=PAREfit2$genes$SYMBOL)

PAREtop100_FOCA <- topTable(PAREfit2, coef="PAREgruposWFOLLOWUP", adjust="bonferroni",
                   number=100)
PAREtop100_FOCA <- PAREtop100_FOCA[,c("SYMBOL","TRANSCRIPT", "ACCESSION","REFSEQ_ID",
                          "ENTREZ_GENE_ID", "PROBE_ID","CHROMOSOME",
                          "PROBE_CHR_ORIENTATION","PROBE_COORDINATES",
                          "logFC","P.Value","adj.P.Val","B")]

write.table(PAREtop100_FOCA,file="PAREtop100_FOCA_030516.txt", sep="\t",
        row.names=F,quote=FALSE)
write.table(PAREresultados,file="PAREresultados_030516.txt", sep="\t",
        row.names=F,quote=FALSE)

#design <- model.matrix(~ID_PEP+B+eri+mono+NK+cd4+gran+cd8)
design  <- model.matrix(~ID_PEP+neutro+NK+monogb+igg+igm+tc+tc_act)
Fit1 <- lmFit(malandro2, design)

correctedValues <- residuals.MArrayLM(Fit1, malandro2)
write.table(correctedValues, file="Playground/Corrigido_FOCA_MDS_cell_020216_GB.txt",
        sep=" ", row.names=F,quote=FALSE)
```

7.6.6 Pré-processamento dos microarrays de metilação e análise de regiões e posições diferencialmente metiladas entre os grupos

```
library(methylumi)
library(TCGAMethylation450k)
library(wateRmelon)
library(minfi)
require(plyr)
library(ggplot2)
library(qqman)
library(DMRcate)
library(IlluminaHumanMethylation450kanno.ilmn12.hg19)
library(RnBeads)
library(limma)
setwd("~/Documents/methylumi/")
## read in 10 BRCA IDATs
idatPath <- system.file("Documents/methylumi/",package='TCGAMethylation450k')
pDat <- read.csv("BR_FEP_Meth_samplesheet_final290116.csv", header=T)
head(pDat)
pDat$barcodes <- as.character(pDat$barcodes)
phenoData <- AnnotatedDataFrame(pDat)
melon <- methylumIDAT(getBarcodes(), pdat=pDat)
class(melon)
melon
dim(melon)
head(betas(melon))
head(pData(melon))
head(fData(melon))
boxplot(methylated(melon))
boxplot(unmethylated(melon))
boxplot(log(methylated(melon)))
boxplotColorBias(melon, channel='methy')
boxplotColorBias(melon, channel='unmethy')
plotColorBias1D(melon)
plotColorBias1D(melon, channel="methy")
plotColorBias1D(melon, channel="unmethy")
plotColorBias2D(melon)
manifest <- read.csv("HumanMethylation450_15017482_v1-2.csv", skip=7)
melon <- melon[-grep("rs", featureNames(melon)),]
melado <- adjColorBias.quantile(melon)
melado_pf <-pfilter(melado,logical.return=T)
gc()
melado_dasen_pf <- dasen(melado_pf)
gc()
boxplot(log(methylated(melado_dasen_pf)))
boxplotColorBias(melado_dasen_pf, channel='methy')
boxplotColorBias(melado_dasen_pf, channel='unmethy')
plotColorBias1D(melado_dasen_pf)
plotColorBias1D(melado_dasen_pf, channel="methy")
plotColorBias1D(melado_dasen_pf, channel="unmethy")
plotColorBias2D(melado_dasen_pf)
pDat2 <- pDat[order(pDat$barcodes),]
row.names(pDat2) <- pDat2$barcodes
CACO <- pDat2[pDat2$CACO == "SIM",]
FOiCA <- pDat2[pDat2$CASE == "SIM",]
FOCA <- FOiCA[FOiCA$FY == "NAO",]
FOLLOWs <- FOiCA[FOiCA$ID %in% names(which(table(FOiCA$ID) > 2)), ]
#FOi <- FOiCA[FOiCA$FY == "SIM",]
betas <- as.data.frame(betas(melado_dasen_pf))
SD <- transform(betas, SD=apply(betas,1, sd, na.rm = TRUE), check.names=F)
Met_vars <- subset(SD, SD > 0.01)
Met_vars <- Met_vars[,1:192]
myMs <- as.matrix(logit2(Met_vars))
myMs.noSNPs <- rmSNPandCH(myMs, dist=2, mafcut=0.05, rmXY= TRUE, rmcrosshyb=T)
nrow(myMs)
nrow(myMs.noSNPs)
#######################################
# CACO
#######################################
Ms_CACO <- myMs.noSNPs[,row.names(CACO)]
#Ms_CACO <- as.data.frame(Ms_CACO)
grupos <- factor(CACO$PHENOTYPE, levels=c("CONTROL", "FEP"))
```

```
sex <- factor(CACO$SEX, levels=c("MALE", "FEMALE"))
age <- CACO$AGE
fuma <- factor(CACO$SMOKE, levels=c("1", "2")) #s?? se era fumante no momento da coleta
fumou <- factor(CACO$SMOKED, levels=c("1", "2")) #EVER SMOKED, n??o estou incluindo, express??o... mais quem est??
fumando.
cor <- CACO$COR
C1 <- CACO$C1
C2 <- CACO$C2
C3 <- CACO$C3
C4 <- CACO$C4
CD8T <- CACO$CD8T
CD4T <- CACO$CD4T
NK <- CACO$NK
Bcell <- CACO$Bcell
Mono <- CACO$Mono
Gran <- CACO$Gran
a <- row.names(CACO)
b <- names(as.data.frame(Ms_CACO))
identical(a,b)
design <- model.matrix(~grupos+sex+age+fumou+C1+C2+C3+C4)
design <- model.matrix(~grupos+sex+age+fumou+C1+C2+C3+C4+NK+CD8T+CD4T+Bcell+Mono+Gran)
myannotation <- cpg.annotate("array", Ms_CACO, analysis.type="differential", design=design, coef="gruposFEP")
dmrcoutput <- dmrcate(myannotation, lambda=1000, C=2, min.cpgs = 3)
results.ranges <- extractRanges(dmrcoutput, genome = "hg19")
results.ranges
results.ranges@ranges@NAMES
cores <- c(FEP="gold", CONTROL="black")
cols <- cores[as.character(grupos)]
DMR.plot(ranges=results.ranges, dmr=1, CpGs=Ms_CACO, phen.col = cols, genome = "hg19", samps=10:20)
resultados_DMR <- dmrcoutput$results
Resultados_DMP <- dmrcoutput$input[order(dmrcoutput$input$fdr),]
Top1000_DMP <- Resultados_DMP[1:1000,]
#Salvando:
write.table(resultados_DMR, file="METIL_Resultados_CACO_DMR_comsangue1205.txt",sep="\t",
row.names=F,quote=FALSE)
write.table(Resultados_DMP,file="METIL_Resultados_CACO_DMP_comsangue1205.txt", sep="\t",
row.names=F,quote=FALSE)
write.table(Top1000_DMP, file="METIL_Resultados_CACO_Top1000DMPs_comsangue1205.txt",sep="\t",
row.names=F,quote=FALSE)
#######################################
# FOCA
#######################################
Ms_FOCA <- myMs.noSNPs[,row.names(FOCA)]
grupos <- factor(FOCA$PHENOTYPE, levels=c("WFOLLOW", "FEP"))
ID <- factor(FOCA$ID)
CD8T <- FOCA$CD8T
CD4T <- FOCA$CD4T
NK <- FOCA$NK
Bcell <- FOCA$Bcell
Mono <- FOCA$Mono
Gran <- FOCA$Gran
a <- row.names(FOCA)
b <- names(as.data.frame(Ms_FOCA))
identical(a,b)
design <- model.matrix(~ID+grupos+CD4T+CD8T+NK+Mono+Gran+Bcell)
design <- model.matrix(~ID+grupos)
myannotation_FOCA <- cpg.annotate("array", Ms_FOCA, analysis.type="differential", design=design, coef="gruposFEP")
dmrcoutput_FOCA <- dmrcate(myannotation_FOCA, lambda=1000, C=2, min.cpgs = 3)
results.ranges_FOCA <- extractRanges(dmrcoutput_FOCA, genome = "hg19")
results.ranges_FOCA
results.ranges_FOCA@ranges@NAMES
cores <- c(FEP="gold", WFOLLOW="orange")
cols <- cores[as.character(grupos)]
DMR.plot(ranges=results.ranges_FOCA, dmr=1, CpGs=Ms_FOCA, phen.col = cols, genome = "hg19", samps=10:20)
resultados_DMR_FOCA <- dmrcoutput_FOCA$results
Resultados_DMP_FOCA <- dmrcoutput_FOCA$input[order(dmrcoutput_FOCA$input$fdr),]
Top1000_DMP_FOCA <- Resultados_DMP[1:1000,]
#Salvando:_FOCA
write.table(resultados_DMR_FOCA, file="METIL_Resultados_FOCA_DMR_comsangue1205.txt",sep="\t",
row.names=F,quote=FALSE)
write.table(Resultados_DMP_FOCA,file="METIL_Resultados_FOCA_DMP_comsangue1205.txt", sep="\t",
row.names=F,quote=FALSE)
write.table(Top1000_DMP_FOCA, file="METIL_Resultados_FOCA_Top1000DMPs_comsangue1205.txt",sep="\t",
```

```
row.names=F,quote=FALSE)
```

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APÊNDICES

9 APÊNDICES

Nessa sessão, serão apresentados, os artigos publicados durante o período do doutorado em que o aluno é coautor.

9.1 **Apêndice 1:**

Accepted Manuscript

Evaluation of neurotransmitter receptor gene expression identifies GABA receptor changes: a follow-up study in antipsychotic-naïve patients with first-episode psychosis

Vanessa Kiyomi Ota , Cristiano Noto , Ary Gadelha , Marcos Leite Santoro , Bruno Bertolucci Ortiz , Elvis Henrique Andrade , Brazilio Carvalho Tasso , Leticia Maria Nery Spindola , Patricia Natalia Silva , Vanessa Costhek Abílio , Marília de Arruda Cardoso Smith , João Ricardo Sato , Elisa Brietzke , Quirino Cordeiro , Rodrigo Affonseca Bressan , Sintia Iole Belangero , Dr

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9.2 Apêndice 2:

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Changes in gene expression and methylation in the blood of patients with first-episode psychosis

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ABSTRACT

Schizophrenia is a severe mental health disorder with high heritability. The investigation of individuals during their first-episode psychosis (FEP), before the progression of psychotic disorders and especially before treatment with antipsychotic medications, is particularly helpful for understanding this complex disease and for the identification of potential biomarkers. In this study, we compared the expression of genes that are involved in neurotransmission and neurodevelopment of antipsychotic-naive FEP in the peripheral blood of patients ($n = 51$) and healthy controls ($n = 51$). In addition, we investigated the differentially expressed genes with respect to a) DNA methylation, b) the correlation between gene expression and clinical variables (PANSS), and c) gene expression changes after risperidone treatment. Expression levels of 11 genes were quantified with SYBR Green. For methylation analysis, bisulfite sequencing was performed. A significant decrease in *GCH1* mRNA levels was observed in FEP patients relative to controls. Also, when we compare the FEP patients after risperidone treatment with controls, this difference remains significant, and no significant differences were observed in *GCH1* mRNA levels when comparing patients before and after risperidone treatment. Additionally, although the differences were non-significant after Bonferroni correction, the expression of *GCH1* seemed to be correlated with PANSS scores, and the *GCH1* promoter region was more methylated in FEP than in controls, thus corroborating the results obtained at the mRNA level. Few studies have been conducted on *GCH1*, and future studies are needed to clarify its potential role in the progression of schizophrenia.

Abbreviations: FEP, first-episode psychosis; PCR, polymerase chain reaction; PANSS, Positive and Negative Syndrome Scale; *GABRR2*, gamma-aminobutyric acid (GABA) receptor, rho 2; GABA, gamma-aminobutyric acid; mRNA, messenger ribonucleic acid; RNA, ribonucleic acid; qPCR, quantitative polymerase chain reaction; SCID, Structured Clinical Interview for DSM-IV; HKG, housekeeping genes; *B2M*, beta-2-microglobulin; *HPRT1*, hypoxanthine phosphoribosyltransferase 1; *RPL13A*, ribosomal protein L13a; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *ACTB*, actin, beta; Ct, threshold cycle; SAGE, serial analysis of gene expression; *GAD67*, glutamic acid decarboxylase 67; *RELN*, reelin; *ABAT*, 4-aminobutyrate aminotransferase; *TSPO*, translocator protein (18 kDa); *CHRNB1*, cholinergic receptor, nicotinic, beta 1 (muscle); *CHRNE*, cholinergic receptor, nicotinic, epsilon (muscle); *COMT*, catechol-O-methyltransferase; *GABRR2*, gamma-aminobutyric acid (GABA)receptor, rho 2; *GCH1*, GTP cyclohydrolase 1; *GCHFR*, GTP cyclohydrolase I feedback regulator; *TACR2*, tachykinin receptor 2; *NRG1*, neuregulin 1.

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9.3 Apêndice 3:

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Effects of depression on the cytokine profile in drug naïve first-episode psychosis

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ABSTRACT

Schizophrenia is accompanied by alterations in immuno-inflammatory pathways, including abnormalities in cytokine profile. The immune assessment of patients in a first episode of psychosis (FEP) and particularly in drug naïve patients is very important to further elucidate this association.
The objectives of this study are to delineate the cytokine profile (IL-2, IL-10, IL-4, IL-6, IFNγ, TNFα and IL-17) in FEP patients (n = 55) versus healthy controls (n = 57) and to examine whether the presence of depressive symptoms in FEP is accompanied by a specific cytokine profile.
We found increased levels of IL-6, IL-10 and TNFα in FEP patients when compared to healthy controls. FEP patients with depression showed higher IL-4 and TNFα levels versus those without depression. Cytokine levels were not correlated to the total PANSS and the positive or negative subscale scores.
Our results suggest that FEP is accompanied by a cytokine profile indicative of monocytic and T regulatory cell (Treg) activation. Depression in FEP is accompanied by monocytic and Th-2 activation, whereas FEP without depression is characterized by Treg activation only. In conclusion, depression emerged as a key component explaining the cytokines imbalance in FEP that is responsible for a large part of the immune–inflammatory abnormalities described.

1. Introduction

Psychosis is a core symptom of schizophrenia, a severe neuro-developmental disorder, with large economical and social impact (Insel, 2010). Abnormal immune–inflammatory responses, including increased levels of pro-inflammatory cytokines are found in patients with schizophrenia (Potvin et al., 2008; Miller et al., 2011). In 1995, the immune–inflammatory theory of schizophrenia proposed that activated immune–inflammatory pathways, particularly activated macrophages and T-lymphocytes, may explain the higher offspring schizophrenia risk associated with gestational infections through the neurotoxic effects of pro-inflammatory cytokines and their detrimental consequences (Smith and Maes, 1995). More recently, several reviews addressed the role of activated immune–inflammatory pathways in the neurodevelopmental pathophysiology of schizophrenia (Anderson et al., 2013a; Meyer, 2013).

Cytokines are proteins involved in the activation, coordination and suppression of immune responses. Their neuromodulatory actions appear to be critical for the regulation of neuroplasticity, cell resilience and apoptosis (Boulanger and Shatz, 2004; Golan et al., 2004; Bauer et al., 2007). Macrophages are activated during innate immune response in two functional distinct states (M1 and M2), producing different cytokines. M1 macrophages produce pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-12 and tumor necrosis factor (TNF)α, stimulating cell-mediated response. M2 macrophages produce negative immunoregulatory cytokines, such as IL-10 and transforming growth factor (TGF)β (Seruga et al., 2008; Maes et al., 2012b). During the adaptive immune response, T lymphocytes differentiate into T helper (Th)1, Th17, T regulatory (Treg) and Th2 cells. Naïve cells are driven towards Th1 and Th2 phenotypes by M1 and M2 macrophages, respectively (Seruga et al., 2008; Maes et al., 2012b). A Th1-shift cytokine profile indicates immune activation, whereas a Th2-shift may indicate negative immunoregulatory effects and activated humoral immunity

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9.4 **Apêndice 4:**

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Depression, Cytokine, and Cytokine by Treatment Interactions Modulate Gene Expression in Antipsychotic Naïve First Episode Psychosis

Cristiano Noto[1,2] &Vanessa Kiyomi Ota[1,3] &Marcos Leite Santoro[3] &
Eduardo Sauerbronn Gouvea[2,3] &Patricia Natalia Silva[3] &Leticia Maria Spindola[3] &
Quirino Cordeiro[1,2] &Rodrigo Affonseca Bressan[1] &Ary Gadelha[1] &Elisa Brietzke[1] &
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Abstract In schizophrenia, genetic and environmental factors affect neurodevelopment and neuroprogressive trajectory. Altered expression of neuro-immune genes and increased levels of cytokines are observed, especially in patients with comorbid depression. However, it remains unclear whether circulating levels of cytokines and expression of these genes are associated, and how antipsychotic treatments impact this association. Relationships between messenger RNA (mRNA) expression of 11 schizophrenia-related genes and circulating levels of cytokines (interleukin (IL)-6, IL-10, and tumor necrosis factor (TNF)-α) were analyzed in 174 antipsychotic naïve first episode psychosis (FEP) and in 77 healthy controls. A subgroup of 72 patients was reassessed after treatment with risperidone. FEP patients were divided into those with and without depression. FEP patients with depression showed increased COMT expression and decreased NDEL1 expression. Increased IL-6 was associated with lowered AKT1 and DROSHA expression, while increased IL-10 was associated with increased NDEL1, DISC1, and MBP expression. IL-6 levels significantly increased the risperidone-induced expression of AKT1, DICER1, DROSHA, and COMT mRNA. The differential mRNA gene expression in FEP is largely associated with increased cytokine levels. While increased IL-6 may downregulate AKT-mediated cellular functions and dysregulate genes involved in microRNA (miRNA) machinery, increased IL-10 has neuroprotective properties. Increased IL-6 levels may prime the expression of genes (AKT1, DICER1, DROSHA, and COMT) in response to risperidone, suggesting that cytokine×treatment×gene interactions may improve cell function profiles. FEP patients with depression show a different gene expression profile reinforcing the theory that depression in FEP is a different phenotype.

Keywords Schizophrenia · First-episode psychosis · Antipsychotic naïve · Neuroprogression · Gene expression · Depression · Inflammation · Immune

Cristiano Noto and Vanessa Kiyomi Ota contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s12035-015-9489-3) contains supplementary material, which is available to authorized users.

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Introduction

Schizophrenia is a complex mental disorder, one of the leading causes of disease burden in the world, notably in young adults [1]. Its phenotype is highly heterogeneous, possibly due to different clinical subgroups [2] and staging characteristics [3]. It has an important genetic component, with a high heritability, estimated at up to 80 % [4], in which common genetic

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9.5 Apêndice 5:

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Low expression of *Gria1* and *Grin1* glutamate receptors in the *nucleus accumbens* of Spontaneously Hypertensive Rats (SHR)

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ABSTRACT

The Spontaneously Hypertensive Rat (SHR) strain is a classical animal model for the study of essential hypertension. Recently, our group suggested that this strain could be a useful animal model for schizophrenia, which is a severe mental illness with involvement of glutamatergic system. The aim of this study is to investigate glutamatergic receptors (*Gria1* and *Grin1*) and glycine transporter (*Glyt1*) gene expression in the prefrontal cortex (PFC) and *nucleus accumbens* (NAcc) of SHR animals. The effects in gene expression of a chronic treatment with antipsychotic drugs (risperidone, haloperidol and clozapine) were also analyzed. Animals were treated daily for 30 days, and euthanized for brain tissue collection. The expression pattern was evaluated by Real Time Reverse-Transcriptase (RT) PCR technique. In comparison to control rats, SHR animals present a lower expression of both NMDA (*Grin1*) and AMPA (*Gria1*) gene receptors in the NAcc. Antipsychotic treatments were not able to change gene expressions in any of the regions evaluated. These findings provide evidence for the role of glutamatergic changes in schizophrenia-like phenotype of the SHR strain.

1. Introduction

The Spontaneously Hypertensive Rat (SHR) strain has been extensively used to study hypertension (Pravenec et al., 2014). This strain was developed in Kyoto, Japan, and is the result of cross-breed Wistar rats (WR) selected with increased blood pressure (Johnson et al., 1992; Louis and Howes, 1990; Okamoto and Aoki, 1963). Noteworthy, the behavioral alterations also presented by SHR animals have supported this strain as a valid model to the study of psychiatric disorders (Calzavara et al., 2009; Russell, 2007; Sagvolden and Sergeant, 1998). In this respect, the hyperactivity, increased impulsivity and sustained attention problems (Russell, 2007) displayed by the SHR strain were initially associated to Attention-Deficit/Hyperactivity Disorder (ADHD) (Sagvolden and Sergeant, 1998). However, the administration of psychostimulants (current treatment to ADHD) is unable to reverse these behavioral abnormalities (Bizot et al., 2007; Calzavara et al., 2009; van den Bergh et al., 2006), indicating a poor predictive validity. Furthermore, WKY rats do not seem to represent the best control for SHR animals, since they display behavioral abnormalities consistent to a depression-like phenotype (Overstreet, 2012; Pare, 1994), as well as a great degree of genetic discrepancy when compared to SHRs (Johnson et al., 1992; Wickens et al., 2011).

More recently, it has been demonstrated that the SHR strain – compared to WR-presents several behavior abnormalities classically associated to schizophrenia animal models, such as deficit in the PPI test, reduced social interaction, hyperlocomotion, and deficit in the contextual fear conditioning (cognitive deficit) that were specifically reversed by antipsychotic drugs and aggravated by pro-schizophrenic manipulations (Calzavara et al., 2011a, 2009, 2011b; Levin et al., 2011). Adding to this behavioral and

Abbreviations: SHR, spontaneously hypertensive rat; Gria1, glutamatergic AMPA-R 1gene; Grin1, glutamatergic NMDA-R 1 gene; Glyt1, Glycine transporter gene; PFC, prefrontal cortex; NAcc, Nucleus accumbens; WR, wistar rats; ADHD, attention deficit hyperactivity disorder; WKY, wistar Kyoto rats; PPI, pre-pulse inhibition; PCP, phencyclidine; NMDA-R, N-methyl-D-aspartate receptor; AMPA-R, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor; GLYT, glycine transporter; CNS, Central Nervous System; Glra1, Glycine receptor 1 gene

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0165-1781/

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Serum brain-derived neurotrophic factor and cortical thickness are differently related in patients with schizophrenia and controls

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) has been implicated in neuronal plasticity, a key process related to the pathophysiology of schizophrenia. However, the relationship of peripheral levels of BDNF to cortical thickness and subcortical structures has not been extensively investigated. This study aims to investigate the relationship of peripheral serum BDNF levels to cortical thickness and volumes of the hippocampus and amygdala. Twenty-nine patients with schizophrenia and 32 healthy controls were included in this study. Structural magnetic resonance imaging (MRI) scans obtained in a 1.5 T scanner were performed in all subjects. Images were processed using Freesurfer software. Blood samples were collected on the same day of the MRI scan for BDNF peripheral levels. Vertex-wise analysis revealed significantly thinner cortex in patients compared with controls. BDNF levels and cortical thickness showed different patterns of correlation for patients and healthy controls in one cluster in the right hemisphere distributed across the supramarginal, postcentral, and inferior frontal cortices.

1. Introduction

Brain-derived neurotrophic factor (BDNF) that has been implicated in neurogenesis, neuron survival, and synaptic plasticity (Vicario-Abejon et al., 2002; Nieto et al., 2013). BDNF is thought to be involved in the pathophysiology of mood disorders, anxiety disorders and psychosis (Kapczinski et al., 2008).

Studies investigating BDNF levels in schizophrenia have found conflicting results (Pedrini et al., 2011; Favalli et al., 2012; Asevedo et al., 2013; Nieto et al., 2013). A meta-analysis by Green et al. (2011) concluded that BDNF levels, despite the somewhat heterogeneous results, tend to be decreased in comparison with levels in healthy individuals. Recently, further studies have indicated a positive association between BDNF levels and clinical features of schizophrenia, such as the severity of negative symptoms (Niitsu et al., 2014).

In schizophrenia findings of decreased cortical thickness and volume relative to controls have been consistently reported. Although there are reductions in volume across many regions of the brain, volumetric reductions in specific cortical and subcortical regions tend to be more prominent (Woods et al., 2005; Haijma et al., 2013;). The actions of neurotrophins have been linked to activity-dependent synaptic plasticity with long-term modulation of synaptic connections (Poo, 2001), suggesting that imbalances of neurotrophin levels or in their molecular actions could be related to structural changes seen in schizophrenia. Nevertheless, in a post-mortem study, Durany et al. (2001) found a significant increase in BDNF concentrations in frontal, parietal, temporal and occipital cortical areas and a decrease in the hippocampus of patients with schizophrenia compared with controls. Another study found that BDNF levels were elevated in the anterior cingulate cortex and hippocampus (Takahashi et al., 2000).

BDNF gene single polymorphisms in the Val66Met have been associated with a heightened risk of developing schizophrenia (Muglia et al., 2003; Neves-Pereira et al., 2005) and with the response to antipsychotics (Zai et al., 2012). Moreover, hippocampal function appears to be affected differently by this polymorphism in patients and controls (Eisenberg et al., 2013). The BDNF

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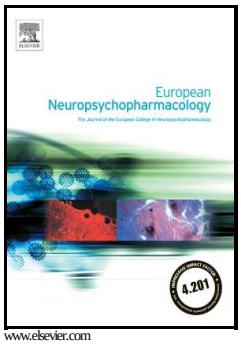
9.7 **Apêndice 7:**

Author's Accepted Manuscript



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Increased expression of *NDEL1* and *MBP* genes in the peripheral blood of antipsychotic-naïve patients with first-episode psychosis

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9.8 Apêndice 8:

Summaries of plenary, symposia, and oral sessions at the XXII World Congress of Psychiatric Genetics, Copenhagen, Denmark, 12–16 October 2014

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The XXII World Congress of Psychiatric Genetics, sponsored by the International Society of Psychiatric Genetics, took place in Copenhagen, Denmark, on 12–16 October 2014. A total of 883 participants gathered to discuss the latest findings in the field. The following report was written by student and postdoctoral attendees. Each was assigned one or more sessions as a rapporteur. This manuscript represents topics covered in most, but not all of the oral presentations during the conference, and contains some of the major notable new findings reported. ***Psychiatr Genet*** **26:1–47**

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*Rapporteurs in alphabetical order, all performed equal work.

†Freida K. Cormack is the coordinator of rapporteurs and this summary.

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Introduction

The XXII World Congress of Psychiatric Genetics, sponsored by the International Society of Psychiatric Genetics (ISPG), took place on 12–16 October 2014, in Copenhagen, Denmark. A total of 883 researchers in the fields of psychiatry, psychology, and molecular genetics attended the conference to discuss the latest technological advances in the field and new findings on the genetics of mental disorders.

The ISPG was first established as a nonprofit corporation in the USA in 1992 and is now a worldwide organization that strives for the highest methodologies to the study of psychiatric disorders. It was formed to provide a stable structure for continual congresses in this field with the mission of overseeing an annual gathering at different international locations. The congress was co-chaired by Dr Ole Mors, Dr Thomas G. Schulze, and Dr Thomas Werge. Rapporteurs for these sessions were students or postdocs who covered the presentations. They were tasked to summarize individual sessions as well as relevant discussions. Similar accounts of the 2007, 2008, 2009, 2010, 2011, 2012, 2013, and 2014 congress held in New

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9.9 Apêndice 9:

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ORIGINAL ARTICLE

Gene expression alterations related to mania and psychosis in peripheral blood of patients with a first episode of psychosis

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Psychotic disorders affect ~3% of the general population and are among the most severe forms of mental diseases. In early stages of psychosis, clinical aspects may be difficult to distinguish from one another. Undifferentiated psychopathology at the first-episode of psychosis (FEP) highlights the need for biomarkers that can improve and refine differential diagnosis. We investigated gene expression differences between patients with FEP–schizophrenia spectrum (SCZ; $N = 53$) or FEP–Mania (BD; $N = 16$) and healthy controls ($N = 73$). We also verified whether gene expression was correlated to severity of psychotic, manic, depressive symptoms and/or functional impairment. All participants were antipsychotic-naive. After the psychiatric interview, blood samples were collected and the expression of 12 psychotic-disorder-related genes was evaluated by quantitative PCR. *AKT1* and *DICER1* expression levels were higher in BD patients compared with that in SCZ patients and healthy controls, suggesting that expression of these genes is associated more specifically to manic features. Furthermore, *MBP* and *NDEL1* expression levels were higher in SCZ and BD patients than in healthy controls, indicating that these genes are psychosis related (independent of diagnosis). No correlation was found between gene expression and severity of symptoms or functional impairment. Our findings suggest that genes related to neurodevelopment are altered in psychotic disorders, and some might support the differential diagnosis between schizophrenia and bipolar disorder, with a potential impact on the treatment of these disorders.

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INTRODUCTION

Psychotic disorders, including schizophrenia, bipolar disorder and schizoaffective disorder, affect ~3% of the general population[1,2] and represent some of the most severe mental diseases. Characteristic symptoms include hallucinations, delusional beliefs, severe mood variations and cognitive impairment. However, during the early stages of psychosis, the clinical aspects may be difficult to distinguish from one another. The first-episode psychosis (FEP) is a critical period given that brain abnormalities and cognitive deficits are already present and progress faster, and more aggressively in the first years of the disorder,[3] whereas the patients are not affected yet by factors related to disease progression, that is, duration of illness and exposure to antipsychotics.[4,5]

More than a century has passed since Kraepelin first proposed the distinction between dementia praecox (schizophrenia) and manic-depressive insanity (bipolar disorder),[6] but as both disorders may share the same psychotic symptoms, differentiating schizophrenia spectrum disorders from bipolar disorder is still a challenge. Therefore, different lines of research aim to identify biomarkers capable of distinguishing these disorders, including studies based on gene expression in peripheral tissues.[7] On the other hand, some genes, including microRNAs, show a concordant expression and association for both schizophrenia and bipolar disorder in blood[8] and also in brain tissues,[9,10] showing a possible common pathophysiological mechanism between these disorders, beyond the diagnostic boundaries. Moreover, previous studies revealed an effect of antipsychotics on gene expression.[11–13] Therefore, assessing gene expression in early stages, such as FEP, is crucial, particularly before the administration of antipsychotics, but this is only feasible in peripheral tissues.

The majority of studies have focused on schizophrenia-spectrum psychosis, suggesting alterations in genes related to myelination, neurodevelopment and AKT pathway,[14] although affective psychoses studies are under-represented in the literature, particularly early-stage affective psychoses. Very few studies on gene expression of antipsychotic-naive bipolar disorder patients have been conducted,[15–18] reporting alterations in inflammatory genes, such as *TNF*,[15] and in genes of AKT1/mTOR pathway.[18]

Our objectives are to investigate differences in the messenger RNA (mRNA) levels of 12 genes among individuals with FEP of schizophrenia-spectrum disorder (SCZ), FEP with mania (BD) and healthy controls. We also want to verify whether gene expression is correlated to clinical features, including functional impairment and severity of psychotic, manic, and depressive symptoms. Particularly, we compared SCZ with BD to identify diagnostic specificity (genes related to manic symptoms), and FEP (both SCZ and BD) and healthy controls to find genes related to psychosis itself as a broad syndrome.

To our knowledge, this is the first study that compares gene expression between antipsychotic-naive FEP of schizophrenia spectrum disorder and FEP with mania. The study aims are to

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